



# Investigating Stem Cell Interactions using Atomic Force Microscopy

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## **Abstract**

Embryonic stem cells (ESC's) hold great promise for future clinical therapies including for tissue replacement due to their remarkable capabilities for self-renewal and pluripotency. While most studies in the field of stem cell research have focused on how biochemical factors, signaling pathways, and transcriptional networks control differential gene expression, few studies have investigated the impact of mechanical signals such as cell adhesion on stem cell behavior.

E-cadherin is a unique target to study the importance of mechanical signals in embryonic stem cell differentiation. Not only is it associated with maintaining the pluripotency of ESC's but it also functions as an adhesive molecule. Atomic force microscopy (AFM) has been shown to be an effective tool to examine the molecular interaction forces between cadherin molecules immobilized on solid surfaces and cells. This thesis thus aimed to explore for the first time E-cadherin interactions on the surface of undifferentiated ESC's using AFM.

First a surface functionalization technique was developed to demonstrate that it was possible to attach recombinant E- cadherin ectodomain constructs to AFM tips and silicon surfaces and to measure their interactions by AFM force measurements. By bringing functionalized AFM tips and surfaces into and out of contact the force necessary to rupture formed E-cadherin bonds, as well as the frequency of bond formations could be quantitatively measured. For comparison the binding behavior of the classical cadherin N-cadherin was also assessed using the same type of experiments. Rupture forces for

homophillic and heterophillic bonds between the E and N cadherin ectodomains had modal rupture forces that were in agreement with forces reported in literature using similar methodology (20-80pN)<sup>12, 15, 117, 118</sup>. E- and N-cadherin bond rupture forces were found to decline with increasing AFM tip speeds.

After having studied cadherin interactions at the single molecule level, the same methodology was used to study cadherin bond formations on mouse embryonic stem cells (mESC's). It was possible to measure bond strengths and frequencies of interactions between the cadherin ectodomain constructs immobilized on AFM tips and native cadherin on the cell surface. E- and N-cadherin bond rupture forces strongly resembled those previously measured in single molecule experiments (i.e.<10 pN difference). In preliminary experiments it was possible also to detect potential differences in E-cadherin interactions when mES cells were allowed to differentiate into precursor cells of the neuronal lineage, suggesting that that a lineage specification of undifferentiated mES cells goes hand in hand with a change of E-cadherin mediated adhesion.

Lastly, a model system based on holographic optical tweezers (HOT) was developed as an alternative approach to AFM to investigate cadherin interactions on mES cells. Optical tweezers possess a unique potential to explore the nature of cadherin interactions, with a greater resolution of detecting bond rupture forces compared to AFM. Although it was possible to investigate interaction forces between trapped silicon beads, E-cadherin coated beads and mES cells, several technical issues were identified in our

experiments; for example the close proximity of optical traps was found to cause interference and resulted in irregularities of force curves.

With further development this approach could be used as a tool to advance stem cell research, by identifying the pluripotent state as well as other stages of development based on physical, rather than by using chemical or genetic cues. This could be of great benefit in cancer research when discriminating between normal and cancerous cells, as well as in regenerative medicine, tissue engineering, drug discovery, disease modelling and developmental biology.

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# **Abbreviations**

| <u>Abbreviation</u> | <u>Explanation</u>                                                   |
|---------------------|----------------------------------------------------------------------|
| μl                  | Micro Litre                                                          |
| μM                  | micro Molar                                                          |
| μm                  | micrometer                                                           |
| Abbreviation        | Explanation                                                          |
| AFM                 | Atomic Force Spectroscopy                                            |
| AJ                  | Adherence Junction                                                   |
| AMAS                | (N-α-maleimidoacet-oxysuccinimide ester)                             |
| CFS                 | Cell based Force Spectroscopy                                        |
| DFS                 | Dynamic Force Spectroscopy                                           |
| EC                  | Extracellular Cadherin Domain                                        |
| CAM                 | Cell Adhesion Molecule                                               |
| EDTA                | (Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) |
| EGTA                | Ethylenediaminetetraacetic acid                                      |
| ESC                 | Embryonic Stem Cell                                                  |
| fN                  | Femtonewton                                                          |
| HOT                 | Holographic Optical Tweezers                                         |
| mES                 | Mouse Embryonic Stem cell                                            |
| MΩcm                | Mega Ohm centimeters                                                 |
| NP                  | Neuronal Precursor Cell                                              |
| OT                  | Optical Tweezers                                                     |
| pN                  | Pico Newton                                                          |
| SMFS                | Single Molecule Force Spectroscopy                                   |

# **Chapter I**

## **Introduction**

### **1.1. Cell adhesion; the foundation of tissue structure**

Without the interaction of proteins any living cell or organism would cease to function. Cell survival is strongly dependent on the precise kinetic and mechanical properties of their adhesion receptors and a rigorous regulation of these molecules <sup>1, 2</sup>. In multicellular organisms cells must be capable of organizing themselves into distinct and highly diverse patterns whilst staying in contact with one another <sup>3</sup>. They form complex structures that can perform the necessary functions to enable them to respond to the environment, to survive and reproduce. Hence the bonds that hold cells together in multicellular organisms are perhaps the most fundamental of all intercellular interactions.

Attachment of cells to other cells within tissue is made possible through cell junctions <sup>4</sup>. These junctions connect cells with one another and with the extracellular matrix and are dependent on the compacted anchorage sites of the cytoskeleton of one cell being linked to the cytoskeleton of another cell. In order to maintain an organized connection of cell assemblies, two main types of tissues can be distinguished: epithelial tissues and connective tissues. This thesis focuses on the study of cadherin interactions which can only be found in epithelial tissues. Therefore explanations of the structure and function of epithelial tissues and especially, those of cadherins, will be given in the sections below.

## 1.2. Epithelial tissues and cadherins

In epithelial tissues, such as the epidermal layer of the skin and the lining of blood vessels and organs, cells are directly linked to one another through cell to cell junctions <sup>4</sup>. These junctions bind cells tightly together by connecting the cytoskeletal filaments of adjacent cells to form a sheet called the epithelium. Within the epithelium, the cells themselves are the main strain bearing entity. The epithelium is connected through cell-matrix attachments to a thin layer of extracellular matrix called the basal lamina. The so called anchoring junctions are cell junctions that connect the cytoskeletons of adjacent cells and are comprised of two main types: the desmosomes which connect the intermediate filaments at distinct anchorage sites and adherens junctions which connect the actin filament contact sites of one cell with that of another cell (Figure 1.1.A) <sup>3</sup>. Adherens junctions depend on trans-membrane adhesion proteins that link the cytoskeleton to structures outside the border of the cell membrane and its lipid bilayer. Proteins of the cadherin superfamily are the main mediators of cell to cell attachment in adherens junctions and are indirectly linked to the cytoskeleton through an intricate complex of adaptor proteins (Figure 1.1.B) <sup>3-6</sup>.

The diagram illustrates the structure of an adherens junction. On the left, the extracellular space contains red, branched structures representing the cell surface. The plasma membrane is shown as a vertical orange bar. Just inside the membrane, a blue oval represents the E-cadherin transmembrane protein. A green line extends from the intracellular tail of E-cadherin, passing through a series of orange circles (representing catenins) and green ovals (representing the actin filament network of the cytoskeleton). The right side of the diagram shows the intracellular space, where the actin filament network is depicted as a red, branched structure. Labels include: "Adherens Junction" (top center), "Intracellular space" (top right), "β catenin α catenin" (top right, pointing to the orange circles), "Ca<sup>2+</sup> Ion" (middle left, pointing to the green line), "E-cadherin (Ectodomain)" (middle left, pointing to the blue oval), "Plasma membrane" (bottom left, pointing to the orange bar), "extracellular space" (bottom center), and "actin filament network of the cytoskeleton" (bottom right, pointing to the red structure).

The diagram illustrates the molecular components of a cell-cell junction. At the top, the **plasma membrane** is shown as a horizontal grey line. Above the membrane, the **cadherin** protein is depicted as a chain of five green oval domains labeled **EC1**, **EC2**, **EC3**, **EC4**, and **EC5**. Below the membrane, the **p120-catenin** (blue) and **β catenin** (light blue) proteins are shown. **p120-catenin** is connected to the **cadherin** protein. **β catenin** is connected to **α catenin** (dark blue), which in turn is connected to the **actin filament** (red). The **actin filament** is shown as a double helix of red spheres. The **vinculin** (yellow) protein is also shown, connected to the **actin filament**. An arrow labeled **Myosin II** points to the **actin filament**.

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### 1.3. Structure and function of cadherins

Cadherins are a large superfamily of single pass trans membrane cell–adhesion molecules. Cadherins were named after their dependence on calcium ( $\text{Ca}^{2+}$ ) ions (calcium-dependent adhesion) <sup>5</sup>. Without calcium in the extracellular medium, cell to cell adhesion mediated by cadherins can no longer hold cells together <sup>3</sup>. The cadherin superfamily can be further subdivided into classical and non-classical cadherins <sup>4</sup>. Classical cadherins are closely related in their structure with a conserved cytoplasmic domain and multiple repeats of  $\beta$ -sandwich EC (extracellular cadherin) domains that adopt a similar fold to immunoglobulin domains (Figure 1.2.A). In general, classical cadherins in vertebrates have extracellular portions that contain five subunits of extracellular glycosylated cadherin domains (see Figure (1.2.B.)).

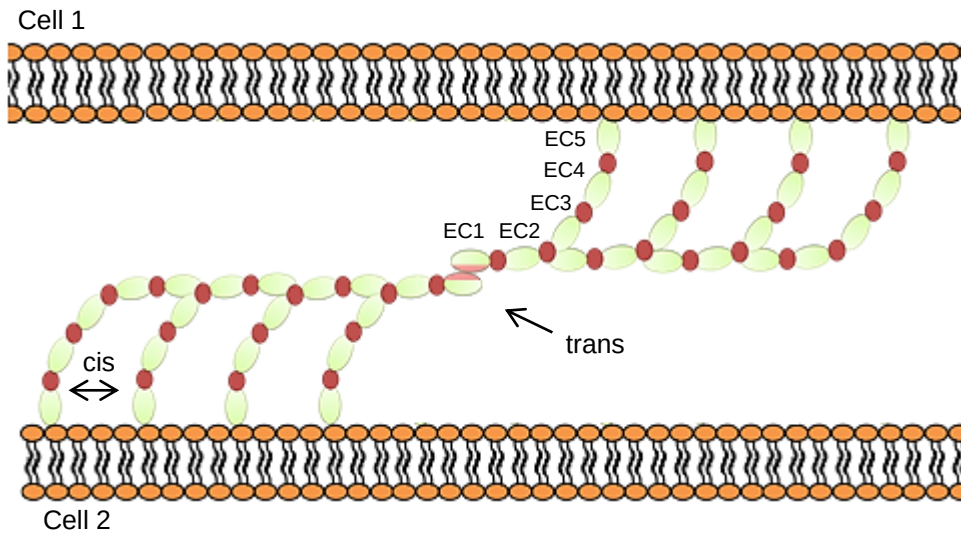
As mentioned above, the intracellular domains of classical cadherins interact with actin filaments of the cytoskeleton <sup>3-6</sup>. The linkage of cadherin ectodomains to the cytoskeleton is indirect and relies on a group of adaptor proteins that assemble around the cytoplasmic tail of cadherin. At adherens junctions the cytoplasmic tail of cadherin is bound to p120 catenin and  $\beta$  catenin. The protein  $\alpha$  catenin interacts with  $\beta$  catenin and recruits other proteins such as vinculin to bind the cytoplasmic tail of cadherin to the actin filaments of the cytoskeleton. In doing this,  $\alpha$  catenin is mediating the mechanotransduction at adherens junctions <sup>4</sup>. These junctions are not passive sites of protein-protein bonds but can be thought of as dynamic tension sensors that are able to trigger adaptive reinforcement of intercellular junctions <sup>3</sup>.

When  $\alpha$  catenin experiences an increase in tension coming from linked cadherin ectodomains between two adjacent cells, non-muscle myosin II pulls from within the cell on actin filaments,  $\alpha$  catenin then unfolds and can bind to vinculin <sup>10</sup>. This in turn reinforces the linkage between the cadherin and the cytoskeleton by recruiting additional actin filaments to bind to the cytoplasmic tail of cadherin. Furthermore  $\beta$  catenin plays an important role in the intracellular signal transduction of the Wnt signalling pathway which is responsible for the activation of target genes such as myc, a gene that is crucial for the promotion of cell growth and proliferation <sup>6</sup>.

The ability of classical cadherins to function as adhesion molecules and to resist detachment force depends, ultimately, on the intrinsic binding properties of their ectodomains. While the structure and function of cadherin has been reviewed extensively in the literature <sup>3-13</sup>, the initial binding mechanisms of cadherins are still a matter of debate <sup>12</sup>. According to the classic model, cadherins first form *cis* dimers between cadherin ectodomains on the same cell (see Figure 1.2.A), then cell adhesion to an opposite cell is achieved by the formation of a low affinity *trans*-adhesive complex between the EC1 domains of cadherin *cis* dimers on opposing cell surfaces <sup>8, 9</sup>. Finally, cadherins cooperatively strengthen the cell adhesion through lateral clustering.

Recent improvements in analytical methods have however provided new data that favour an alternative model of cadherin interactions according to which *cis* dimerization is no longer required for *trans* interactions<sup>10-13</sup> (Figure 1.2.B). This model suggests that the EC1-EC2 subdomains of two cadherins engaged in a *trans* interaction form a fast binding X dimer which then facilitates the formation of a more stable *trans* swapped dimer (Figure 1.3.A). Inside the structure of this *trans* dimer, tryptophan 2 (Trp 2, W2) inserts into the hydrophobic pocket of the EC1 subdomain in an opposing cadherin molecule while interacting laterally with the EC2/EC3 domains of an adjacent cadherin, forming a *cis* dimer and then with increasing numbers of participating cadherin ectodomains, a cadherin lattice (Figure 1.3.B)<sup>12</sup>. It was demonstrated that the N-terminal EC1 subunit of classical cadherins is of special significance during the initiation and specific recognition of cadherins as they direct cell-cell adhesion<sup>14</sup> (Figure 1.4.A). Furthermore, the most recent evidence points towards a rapid, initial formation of *trans* bonds between EC1 domains that facilitates cadherin accumulation at cell-cell junctions, and then, a subsequent slower lateral oligomerization via EC3 domains caused by allosteric alterations which are triggered by the binding event of the EC1 domain<sup>12, 16</sup>. A complete explanation for the fundamental mechanics of these molecular processes is however not yet possible. Nevertheless an understanding of the various aspects that contribute to overall cell adhesion will undoubtedly provide more comprehensive insight into adherens junction mediated cell adhesion.

# A



# B

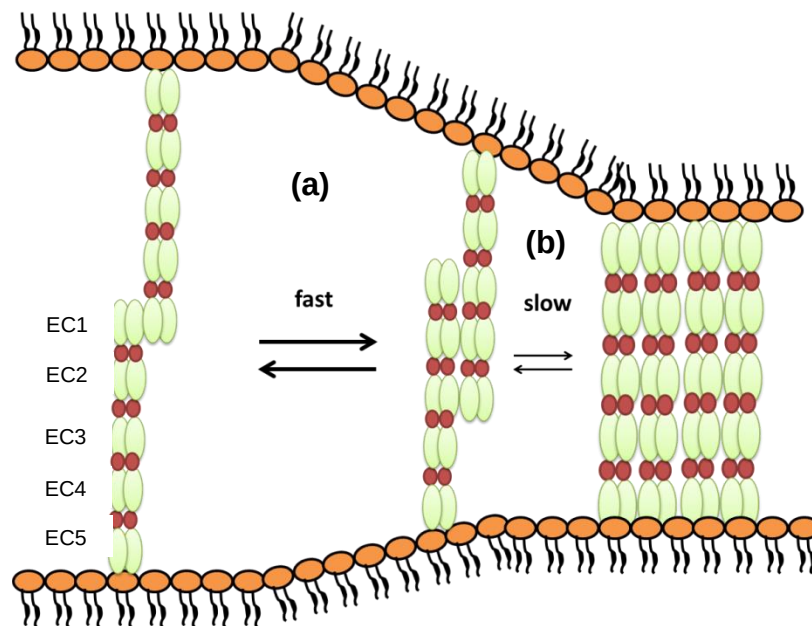


**Figure 1.2. (A)** Schematic depiction of *cis* and *trans* interactions between E-cadherin ectodomains on the cell surface<sup>12</sup>. *Trans* interactions are mediated by the EC1 and occur between cadherin molecules of neighbouring cells. Lateral *cis*-interactions occur between the EC1 of one cadherin molecule and the EC2, the EC2/EC3 linker region and part of the EC3 of an adjacent cadherin molecule exposed on the same cell. A combination of *cis*- and *trans*-interactions is depicted on the right hand side of the scheme. Here, the cadherin ectodomain is simultaneously engaged in a *cis*-interaction with an adjacent cadherin molecule from the same cell, as well as in a *trans*-interaction with a cadherin molecule from an opposite cell (the formation of the fast forming “X dimer” is indicated by the red colour on the EC1 engaged in a *trans* interaction with of an opposing EC1 subunit. **(B)** Model for cadherin junction assembly<sup>15</sup>. **(a)** Cadherins rapidly bind via their outer domains and kinetically discriminate between different cadherins. **(b)** Once the proteins are engaged, the cell membranes are held in close proximity sufficiently long for the slower, strong bonds to form between overlapping proteins. In this cadherin junction assembly, the sequence of binding events is governed by the hierarchy of kinetic rates.

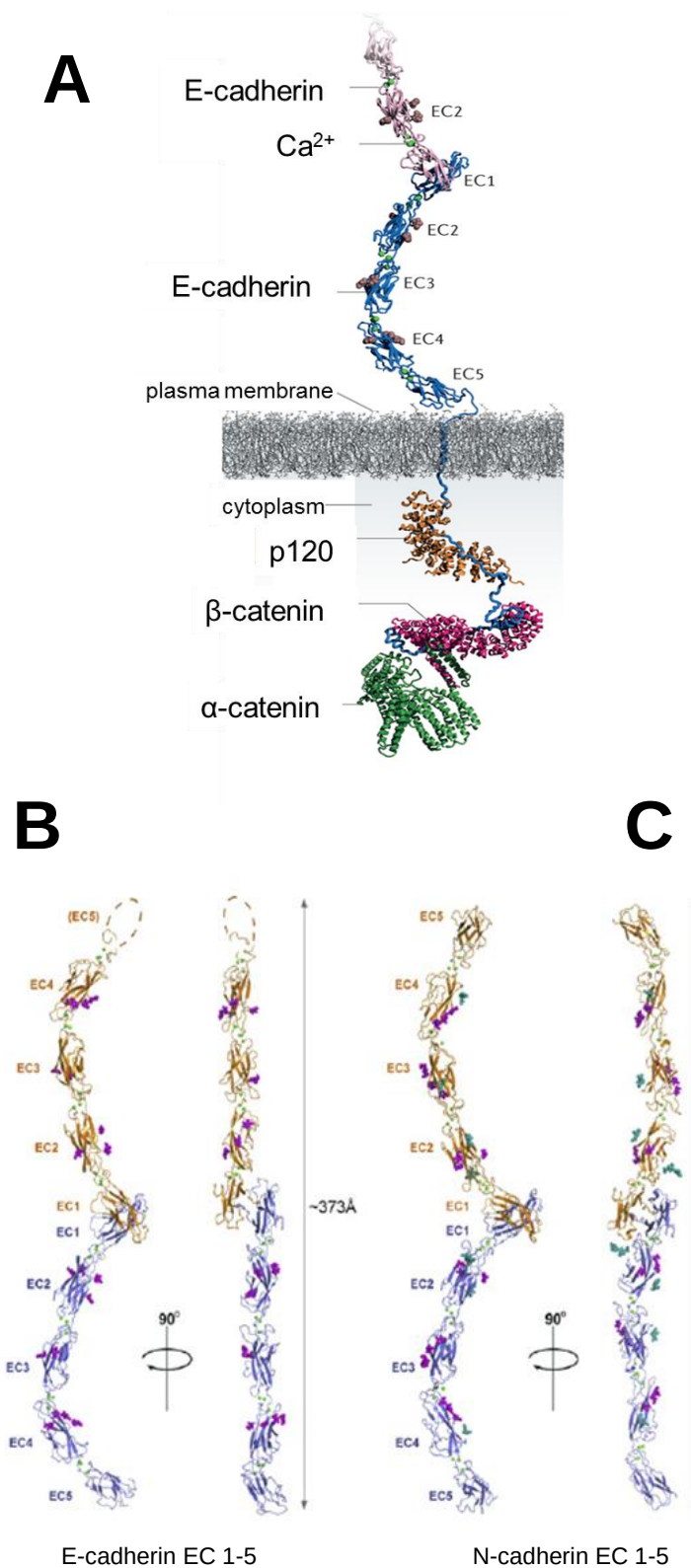


Figure 1.3. (A) Crystal structure of the cadherin–catenin complex <sup>7</sup>. The strand-swapped *trans* dimer of the E-cadherin ectodomain (light pink and blue) is shown with bound  $\text{Ca}^{2+}$  ions (green spheres), PDB-ID:3Q2V. (B) E-cadherin crystal structure showing a strand-swapped *trans* dimer <sup>9</sup>. The opposite E-cadherin ectodomain is coloured in orange. Bound calcium ions are shown as green spheres. Strand-swapped dimerization occurs between EC1 domains and by exchange of Trp2 residues. O-linked glycosylation observed in the structure is shown as magenta spheres. The EC5 domain of the orange coloured cadherin was not resolved and is represented by a dotted line, PDB-ID:3Q2W. (C) N-cadherin strand swapped dimer crystal structure, shown in the same style as for E-cadherin in (B), PDB-ID:3Q2W.

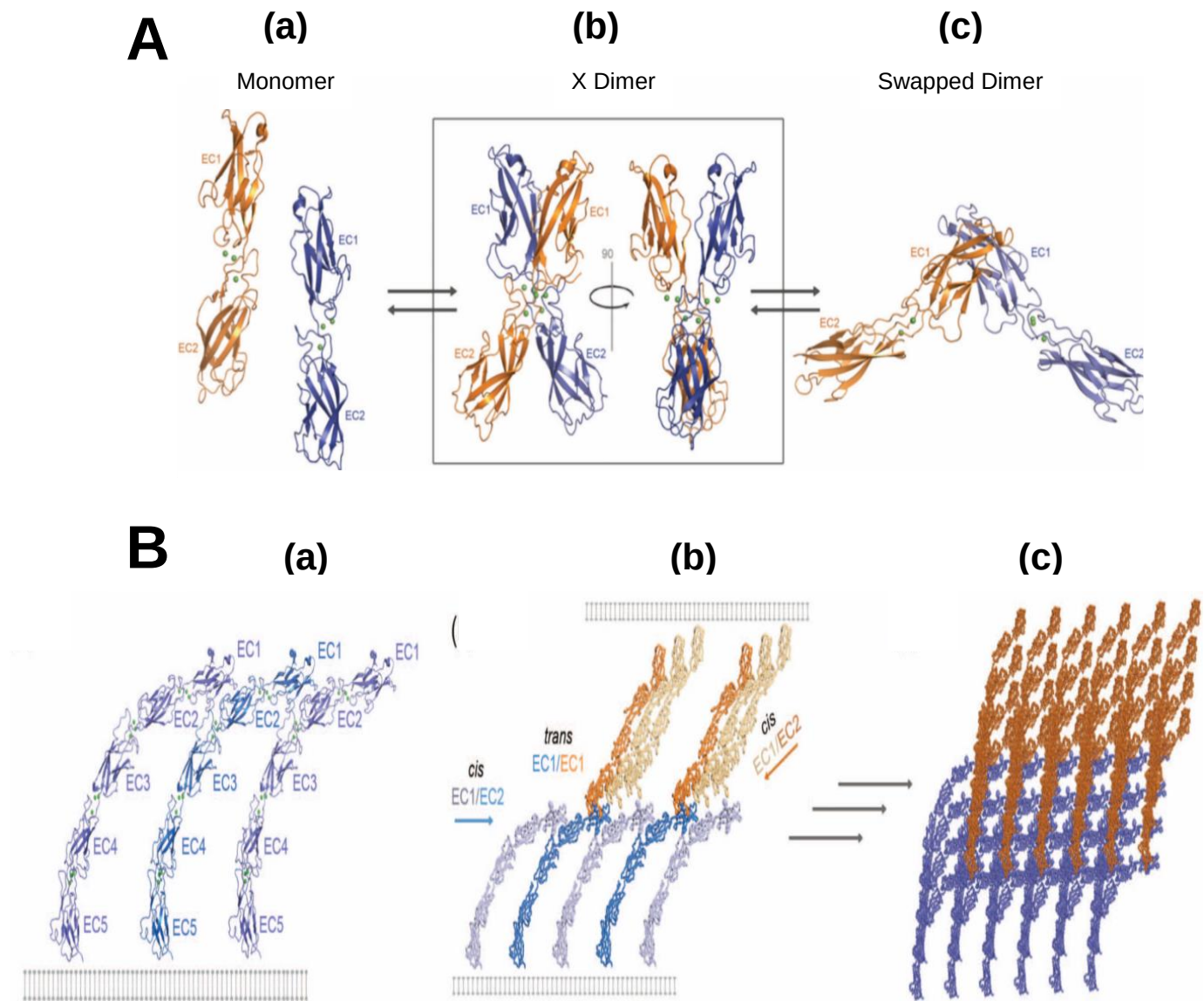


Figure 1.4. (A) E-cadherin EC1-2 associate via an 'X-dimer' interface <sup>9</sup>. (a) N-terminal strands are initially not swapped but are closely apposed. (b) Then swapping of strands leads to formation of a mature strand swapped dimer. (c) Assembly and disassembly of swapped dimers is likely to proceed via the same pathway, PDB-ID:3Q2W. (B) Extracellular structure of adherens junctions formed through *cis* and *trans* interactions of cadherin ectodomains. (a) N-cadherin EC1-5 crystal lattice of an array of N-cadherin molecules emanating from the same cell membrane and connected by a *cis* interface formed between EC1 and EC2 domains of neighbouring molecules. (b) Strand swapped *trans* dimers form together with *cis* interactions in the same crystal lattice. *Trans* interactions orient opposing *cis* arrays approximately perpendicular such that each *cis* array (blue) forms *trans* interactions with multiple opposing *cis* arrays (orange). (c) The combination of *cis* and *trans* interactions enables cadherin ectodomains to form an ordered network that is thought to be the basis for the extracellular architecture of adherens junctions <sup>5</sup> PDB-ID:3Q2W.

## 1.4. Cadherin expression and function in development

Cadherins play a crucial part in cell sorting processes during embryonic development. In general, each subtype of classical type I cadherins is characterized by a unique tissue distribution pattern, amino acid sequence and immunological specificity <sup>3, 10</sup>. Cadherins are essential tools of embryonic morphogenesis because they can direct cell-cell adhesions using a wide variety of different mechanisms to direct tissue morphology, some of which include: expression of different cadherin subtypes, quantitative differences in cadherin expression level, combinatorial expression of cadherin subtypes on the same cell, combination of cadherin subtypes in heterophillic *cis*-dimers, heterophillic interaction of different cadherin subtypes in *trans* configuration and interactions of cadherins with non-cadherin molecules (heterotypic interactions <sup>5</sup>.

The impact of each of these factors is hard to estimate with the current level of information on cadherin binding available, however it is plain to see that the cadherin binding complex has the potential to create and maintain highly intricate and complex cell assemblies such as those found in the vertebrate nervous system <sup>3</sup>. By directing cell adhesions, individual cadherin subtypes mediate selective cell recognition events during development and drive cell sorting in developing tissues <sup>5, 10</sup>.

Differential cadherin expression is believed to determine the differentiation of specific tissue types in morphogenetic processes, such as cell sorting, cell rearrangements, cell movements, cell polarity, invaginations and tube formations <sup>17</sup>. For example, during development a switch from E-cadherin to N-cadherin is associated with mesenchymal epithelial transitions (MET) in which unattached mesenchymal cells such as fibroblasts can come together to form thin layers of tissue known as an epithelium. This is a reversible process and cells of the epithelium can change their characteristics, disassemble and migrate away from their original cell layer as separate cells. This epithelial mesenchymal transition (EMT) also occurs as pathological events, for example in invasive tumor progression in adult life <sup>4</sup>. A similar conversion from E-to N-cadherin can be observed during neurulation, in which the neural tube detaches from the overlaying ectoderm during organogenesis <sup>18</sup>. Here cells that are going to form the neural tube lose E-cadherin expression and acquire the expression of other cadherin types such as N-cadherin, while cells of the overlaying ectoderm continue to express E-cadherin and are retained in their position. Furthermore classical cadherins such as E- and N-cadherin are important in the organization and function of the nervous system. Here they help to maintain synaptic plasticity as well as neurite outgrowth and guidance <sup>17</sup>.

Loss of a tight control of cell-to-cell connectivity mediated by cadherins is one of the main causes for a variety of diseases such as tumour growth <sup>19</sup> and many inherited disorders <sup>20</sup> such as cardiomyopathies <sup>21</sup>, sensory defects leading to blindness <sup>22</sup> and deafness <sup>23</sup>, defect of hair <sup>24</sup> and skin follicles <sup>25</sup> as well as psychiatric disorders <sup>26</sup>. Homophilic N-cadherin interactions are also

of paramount importance in maintaining the structural integrity of the heart <sup>27</sup>. Specifically N-cadherin connects the muscle cells that make up the cardiac muscle tissue. In addition, loss of the gene coding for N-cadherin (*CDH2*) results in disassembly of the end to end connection between cardiac myocytes, called the intercalated disc, leading to cardiomyopathies and impaired cardiac function <sup>28</sup>.

### **1.5. mES cells and cadherins**

Pluripotent embryonic stem cells (ESC's), such as mouse embryonic stem cells (mESC's), have the capacity to differentiate into a wide range of different cell types i.e. cells derived from any of the three germ layers (ectoderm , mesoderm and endoderm) <sup>29</sup>. At the same time ESC's can be maintained in their undifferentiated state in cell culture populations that can be grown indefinitely in the laboratory <sup>30</sup>. Due to these capabilities embryonic stem cells are one of the main objects of research in regenerative medicine, tissue engineering, drug discovery, disease modelling and developmental biology <sup>31</sup>. Despite their great potential, however, ESC's, thus far have had only few clinical applications due to their unique sensitivity to cell culture conditions. Challenges such as undefined chemical culture conditions, heterogeneity of differentiation in EB (embryoid bodies), their survival rates, as well as the cloning efficiency of fully dissociated single ESC's have made it difficult to maintain and expand ESC's and to efficiently direct their lineage differentiation <sup>29-32</sup>. Therefore controlled, reproducible, and fully scalable cell culture methods that yield defined populations ESC's could not yet be established. This can largely be attributed to the relatively unexplored field of physical

interactions of ESC's with their local cellular environment such as dynamic interactions with the ECM or other ESC's <sup>32</sup>. It is therefore essential to consider and understand the mechano-sensitive and-responsive properties of ESC's in order to fully appreciate their unique needs with regards to their culture environment.

Most of the therapeutic applications, as well as the research into the mechanobiology of ESC's has been so far focused on adult stem cells <sup>29</sup>. These cells are multipotent stem cells, which means that they can develop into more than one cell type, but are more limited than pluripotent cells and their function is to maintain and repair the tissue in which they are found. Examples of adult stem cells include stem cells of the bone marrow, mesenchyme or endothelium. There are several limitations to the usefulness of adult stem cells <sup>30</sup>. They are often present in only minute quantities and can therefore be difficult to isolate and purify. Furthermore adult stem cells may not have the same capacity to multiply as embryonic stem cells and may contain more DNA abnormalities caused by sunlight, toxins, and errors in making more DNA copies during the course of a lifetime.

The main focus of studies investigating mechanical signals in embryonic stem cell differentiation have been the effects of substrate stiffness, cell–ECM interactions, cell–cell interactions, and externally applied physical forces <sup>32</sup>. Hammerick *et al.* used AFM as a nanoindenter for determining the elastic modulus of fibroblasts and adipose-derived stromal cells and compared it to that of induced pluripotent stem cells (iPSC) which were derived from these cell types as well as to human embryonic stem cells <sup>33</sup>. They found that the differentiation state of the cell inversely correlated with cell stiffness. Tan *et al.*

used optical tweezers to probe mechanical stiffness of human ESC's (hESC) by attaching two polystyrene beads on either side of a stem cell and applying laser tweezers traps on both beads in order to stretch the cell <sup>34</sup>. They found that committed cardiomyocyte progenitor cells possessed an overall higher degree of stiffness compared to uncommitted hESCs. Other methodological approaches employed to study the mechanical properties of ESCs include micro rheology studies in which the mean squared displacement (MSD) of fluorescent beads inside the cytoplasm were tracked using confocal laser scanning fluorescence microscopy <sup>22</sup>. Related approaches to this are traction force measurements. Here cells are grown onto well characterised hydrogels which are embedded with fluorescent beads. The forces exerted by the cells onto the gel can be measured by tracking the displacement of the beads inside the gel <sup>36</sup>. Furthermore the roughness <sup>37</sup> and stiffness <sup>32</sup> of the substrate on which ESC's are grown has been shown to direct stem cell fates.

Engler *et al.* showed that mesenchymal stem cells differentiated preferentially into a particular lineage when they were grown on a substrate whose modulus matched that of the tissue where the cell type of interest can be typically found <sup>38</sup>. Another way to apply mechanical strain to stem cells is by seeding them onto flexible membranes and then stretching that membrane. Shimizu *et al.* show that cyclic strain induced in this way caused mES cells to differentiate into vascular smooth muscle like cells that expressed many marker genes of this cell type such as smooth muscle  $\alpha$ -actin <sup>39</sup>. The mechanoregulation of stem cell pluripotency has only now begun to be explored, and the current understanding of the mechanotransductive systems in ESC's is still very limited.

One of the main obstacles of implementing stem cells based therapies is the immaturity of ESC's which causes rapid loss of biological function and tumour formations <sup>32, 40</sup>. In order to overcome these obstacles, therefore, it is of paramount importance to be able to distinguish ESC's from other cell types, especially tumour cells. A wide range of cell surface markers and generic molecular markers have been reported to be indicative of undifferentiated ESC's <sup>40-44</sup>. Membrane proteins such as cell adhesion molecules, or CAMs are the most important marker type in recognizing ESC's without breaking the cell membrane. CAM's proteins on the surface of mammalian cells contribute to cell-cell binding or cell-ECM adhesion <sup>45</sup>. Key members of CAMs expressed on mES cells include the Stage Specific Embryonic Antigens (SSEA), Clusters of Differentiation (CD) Antigens, integrins, proteoglycans, the immunoglobulin (Ig) superfamily, and cadherins <sup>40</sup>. The classic cadherin E-cadherin is widely expressed on pluripotent mES cells when the cells are in close contact with each other in undifferentiated colonies, while other classical cadherins such as N-cadherin and VE-cadherin have low expression levels or are not expressed on undifferentiated mES cells <sup>46-55</sup>. Furthermore, E-cadherin functions as a mediator in signal transduction as well as an adhesive molecule and controls the cytoskeleton indirectly through its link to  $\beta$ -catenin <sup>10</sup>. Therefore, E-cadherin could be key, in order to characterize and/or manipulate the differentiation state of embryonic stem cells without having to damage the cell.

## 1.6. Aims

While the structure and function of cadherins has been established the mechanism with which they mediate early embryonic development remains elusive <sup>4,10</sup>. This thesis therefore aimed to begin to address this by characterising interactions of cadherins on the surface of undifferentiated mES cells using AFM, and to investigate how these interactions change as these pluripotent cells differentiate into distinct precursors of the neuronal lineage. We focused on the membrane protein E-cadherin as it can be found on undifferentiated mES cells and is a key mediator of cell adhesion during development <sup>10, 40</sup>. We aimed also to investigate the use of laser tweezers as an alternative force measurement approach to AFM, to investigate cadherin interactions at higher force sensitivity.

These objectives were realized in four distinct steps that demonstrated key feasibilities of our experimental approach. Before describing this work, Chapter II of this thesis presents, the principles of force measurements with an emphasis on AFM, optical tweezers (OT) and dynamic force spectroscopy (DFS) as tools to study biomolecular interaction events. In Chapter III the experimental results that demonstrate our ability to immobilise functional cadherins on surfaces and measure their interactions by AFM are then presented. In the approach described, the extracellular domains of either E or N-cadherin were functionalized to the tip of an AFM cantilever and to the surface of silicon wafers. By bringing the functionalized AFM tip and silicon surfaces into and out of contact, specific interactions between cadherin ectodomains were probed. Chapter IV describes the approach used to show the presence of both E and N-cadherin on the cell membrane of

undifferentiated mES cells and progenitor cells at different time points along the neuronal differentiation pathway. This was achieved by labelling them and quantifying their expression level using immunohistochemistry (IHC). Chapter V, then describes how the approach used in Chapter III was extended to study cadherin interactions with native cadherin expressed on mES cells or neuronal precursor (NP) cells using AFM. Finally, Chapter VI describes our attempts to develop a holographic optical tweezers (HOT) based system to study cadherin interactions. Here, HOT provided the potential to explore the nature of cadherin interactions with a greater sensitivity of detecting rupture forces compared to AFM.

# **Chapter II**

## **Instrumental Background**

### **2.1. Measuring the forces that control protein interactions**

Molecular interactions underpin many of life's most basic processes such as ligand receptor binding, protein and RNA folding and the transcription of genes encoded within DNA <sup>1, 3</sup>. Many of the bonds that are involved in such interactions are weak non covalent forces that include van der Waals forces, hydrogen bonding, entropic and hydrophilic interactions and dipole-dipole binding <sup>4</sup>. One way to explore molecular interactions is by looking at how they respond to an externally applied force. Methods that are able to do this are shear flow detachment techniques <sup>2, 56</sup> and micropipette aspiration <sup>57</sup> as well as instruments such as the surface force apparatus (SFA), bio membrane force probe (BFP), holographic optical tweezers (HOT) and atomic force microscopy (AFM) <sup>58</sup>. Among these techniques AFM is the most widely available method and represents an excellent combination of simple and rapid sample preparation and a broad range of applicable and detectable forces (10 pN to 10<sup>4</sup> pN) <sup>58-61</sup>. In contrast, HOT excel with a high force detection sensitivity (0.1 pN to 100 pN) and ability to manipulate cells and molecules in solution <sup>58, 61</sup>. Both of these techniques were employed within the work of this thesis. In the following chapter the principles of molecular force measurements will be introduced with emphasis on AFM, HOT and dynamic force spectroscopy (DFS).

## 2.2. Single Molecule Force Spectroscopy

Force measurements provide an effective tool to quantify the activity and interactions that determine the function of biological molecules by looking at how they respond to an externally applied force <sup>1, 57</sup>. The basic concept of force experiments is to attach biomolecules to a force probe and a flat surface. These two are brought into contact and are then withdrawn and any adhesion formed is measured. If appropriate control experiments are implemented, this adhesion can be attributed to the interaction of the biomolecules attached to the surfaces <sup>63</sup>.

Some of the first experiments in this field of research were published in 1994 by Florin <sup>65</sup>, Lee <sup>66</sup> and Moy <sup>67</sup> and focused on measuring the interaction between biotin, a small ligand (vitamin H) and its receptor (avidin or streptavidin) using the AFM. These initial pioneering experiments were followed by molecular recognition force experiments of many other ligand-receptor systems, such as between complementary DNA nucleotides (1994) <sup>68</sup>, antibodies and their antigens (1996) <sup>69</sup>, as well as intramolecular forces within polymers and folded proteins (1997) <sup>70</sup>. Furthermore it was discovered that nm or  $\mu$ m sized objects such as small cantilevers <sup>71</sup>, optically trapped beads <sup>72</sup> or magnetically trapped beads <sup>73</sup> could be used as force probes to study ligand-receptor complexes with unprecedented temporal and spatial resolutions. The disruption of chemical bonds formed between biological molecules through application of force using the energy stored in the flexed force probe is known as single-molecule force spectroscopy (SMFS) and is recorded in the form of so called force curves. Force curves are measurements of force per distance cycles where the probe is first moved towards the sample surface whereupon a single receptor–ligand complex is formed, due to the specific ligand receptor recognition. During subsequent probe–

surface retraction a temporary rise in force can be detected. This force is the force exerted on the ligand–receptor connection thus reducing its lifetime until the interaction of the bond breaks at a critical force (the unbinding force)<sup>74</sup>. To ensure that only single molecular bonds are measured the surface density of the molecules under investigation, should be sufficiently low. Usually many unbinding force measurements are made by recording the probe surface approach and retraction several hundred times on different locations on the sample. The measured unbinding force values are then analysed as force histograms, showing the distribution of at least 100 unbinding force values. The reliability and reproducibility of the measured unbinding forces can be demonstrated by measuring the interaction as many times as possible and comparing the data obtained using many independent surface samples and different probe types or force spectroscopy methods. As mentioned above the SMFS techniques used to generate data for this thesis were atomic force spectroscopy (AFM) and holographic optical tweezers (HOT). In order to familiarise the reader with these measurement techniques, their operating principles and practical implementations will now be explained below.

### **2.3. Atomic Force Microscopy (AFM)**

Since its introduction in 1986 AFM has found numerous applications in biology and has opened the door for unique enquiries into the structure and function of biomolecules<sup>74-80</sup>. It allows the measurement of forces with picoNewton detection sensitivity<sup>57</sup>. In single-molecule force measurement studies, AFM tips are commonly functionalized with a small amount of probe molecules (Fig.2.1.A).

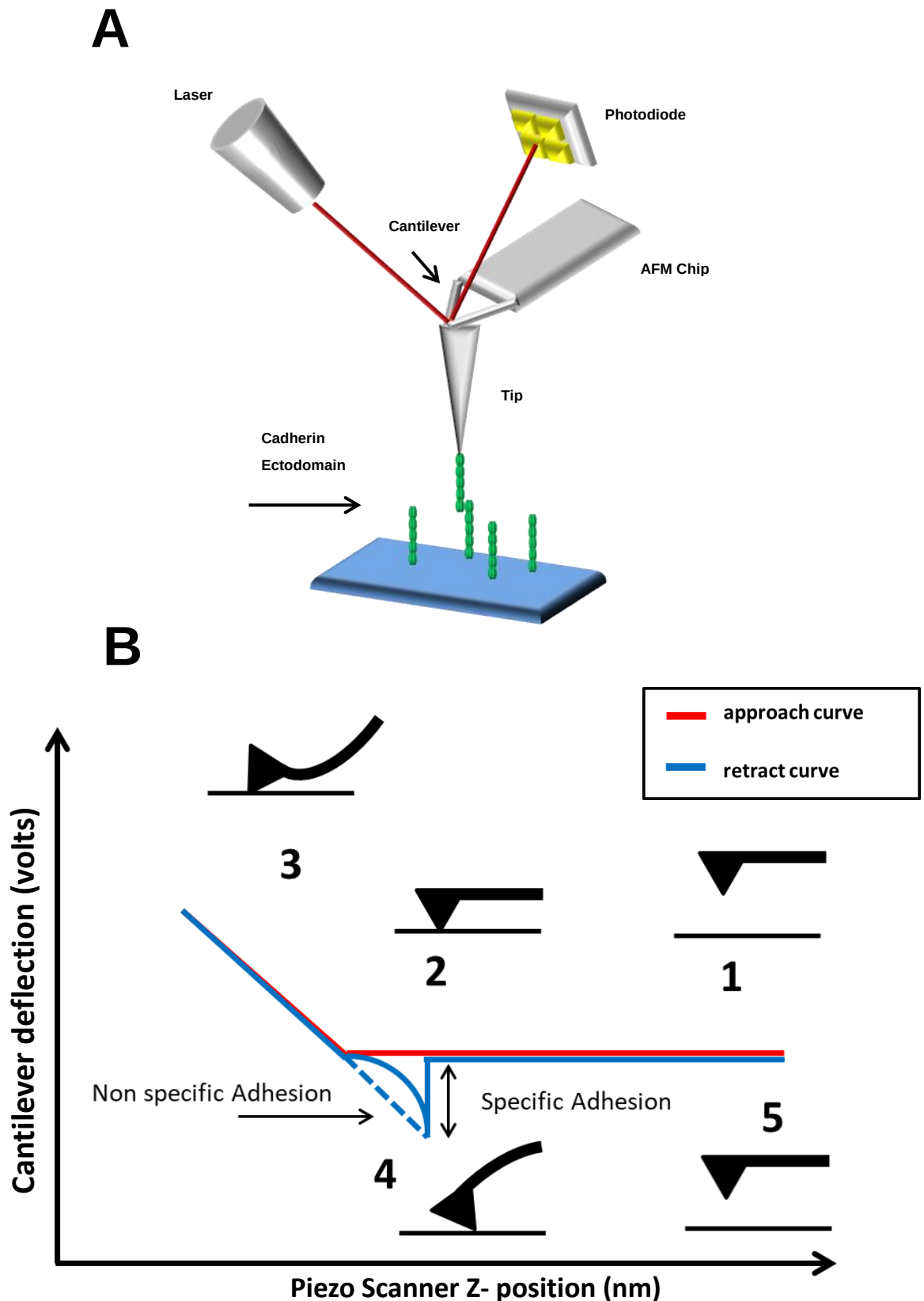


Figure 2.1. (A) AFM Experimental set up employed to measure rupture forces of cadherin bonds. Cadherin ectodomains were attached to AFM tips and silicon surfaces (B) Progression of an AFM force measurement: (1) Approach (2) Contact (3) Deflection (4) Retraction and adhesion (5) Loss of contact. Caution must be taken to correctly discriminate correctly between specific and non-specific adhesion (see Chapter III). The dashed line above indicates non-specific interaction whereas the solid curved lined indicates a specific adhesion event.

These molecules can be recognized by a specific target molecule immobilized on an opposing sample surface. To quantify the adhesion force between the molecules of interest, the functionalized AFM tip is brought into contact with the surface and retracted until it, again loses contact. The cantilever deflection as a function of the vertical displacement of the piezoscanner is recorded and converted into a force– distance curve (Fig.2.1.B). The adhesion forces measured depend on the strength of the molecular bonds formed during tip-surface contact, the measurement speed and for how long the AFM tip is in contact with the sample surface <sup>81</sup>.

For the force measurements carried out for the work of in this thesis, AFM tips and silicon surfaces were functionalized with the extracellular domain of either E- or N-cadherin. The force that is required to bend the AFM cantilever is given by Hooke's Law <sup>82</sup>:

$$F = -kx$$

Equation 2.1.

Where the force (F, N) is directly dependent on the spring constant or stiffness of the cantilever (k, N m<sup>-1</sup>) and the cantilever deflection (x, m). Figure 2.1.B shows a typical progression of a force measurement. First the AFM tip approaches the surface, as represented by the red line (1). When the AFM tip makes contact (2) with the surface the cantilever starts to bend and the deflection increases (3) corresponding to a repulsive force. After a set deflection value is reached the AFM tip stops advancing and retracts as shown by the blue line (4). As the AFM tip moves away from the surface, the cantilever deflection decreases (5). Eventually the AFM tip is retained on the surface by adhesion between AFM tip and surface, and bends the cantilever

downwards (corresponding to an attractive force). When the adhesion is broken the cantilever returns to its default orientation of zero deflection and the AFM tip is retracted away from the surface to its initial position.

The precise detection of the AFM cantilever deflection depends on the correct calibration of the AFM cantilever spring constant and the laser- photodiode sensitivity. The sensitivity is first determined by moving the cantilever a known distance against a hard surface and measuring the photo detector output <sup>81</sup>. The ratio between the cantilever movement and the photo detector output is measured in ( $\text{nm V}^{-1}$ ) and commonly referred to as inverse optical lever sensitivity (InvOLS) <sup>83</sup>. With this information the spring constant of the AFM cantilever ( $k$ ) can be determined using the instrument's built in software, using the thermal noise method <sup>84</sup>. This method relies on the collection of a frequency spectrum of the thermal fluctuations of the cantilever over time.

When an AFM tip and a surface which have been previously functionalized with proteins of interest come into contact with one another, the molecules on their surface can bind to each other. The adhesion force that is recorded during such an AFM measurement is the breakage of the bonds formed between those proteins during contact between the AFM tip and the surface.

In order to ensure that only specific and single bond interactions are observed, the density of the molecules of interest should be kept sufficiently low. Furthermore the bond between the protein and the substrate or the AFM tip to which the protein is attached must be stronger than the intermolecular interaction under investigation. If the frequency of observing an adhesion force is equal or less than 10% it follows that 95% of these events are then due to the rupture of a single molecular, bond according to Poisson statistics

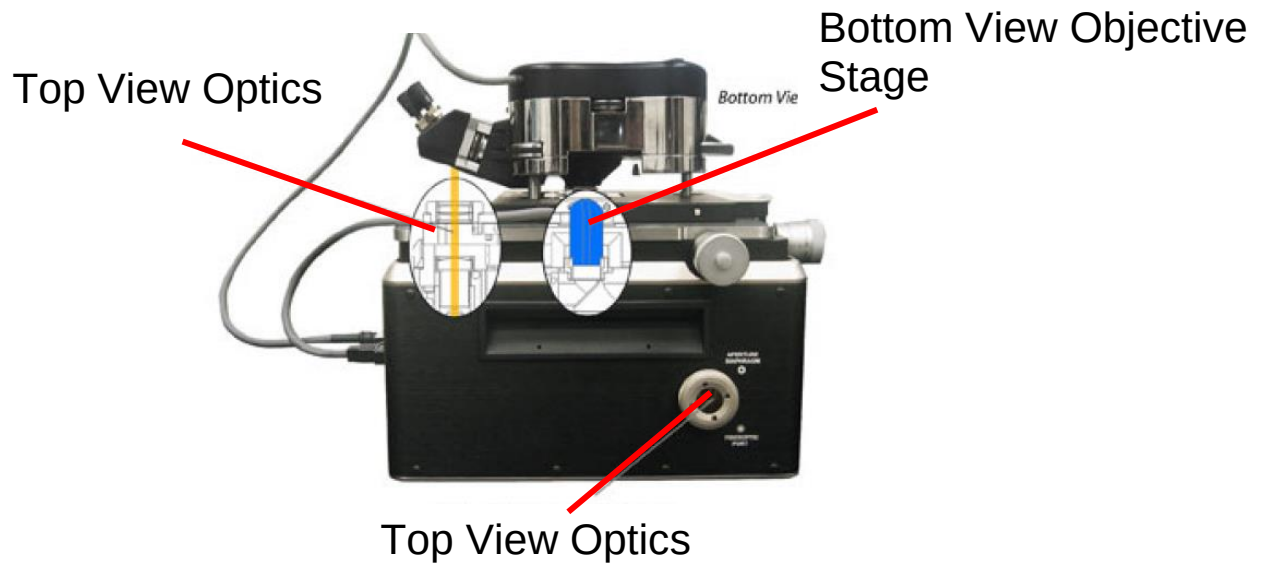
<sup>85, 86</sup>. Appropriate criteria and control experiments must be put in place to ensure that only specific rupture events are considered for data analysis (see Figure 2.1.B) i.e. interactions between of the molecules of interest.

The AFM instrument that was used for the work presented in this thesis was the MFP-3D Atomic Force Microscope from Asylum Research <sup>87</sup>. The Asylum Igor pro software was used for analysis and display of the force curve data. Figure 2.2. shows the basic components of the MFP 3D overhead that is mounted on the optical platform. The MFP is a single axis force curve tracer designed specifically for soft samples, with picoNewton force measurement capability and was fitted with an high speed Video-AFM imaging facility capable of operation in liquid and ambient conditions. These properties made the MFP 3D ideal for working with biological macromolecules such as cadherins and working with cell samples such as the embryonic and neuronal cell specimens used for the work presented in the later chapters of this thesis as these proteins and cells need to be in a liquid environment to function and survive.

## **2.4. General overview of Optical Tweezers**

In order to support our force measurements made with the atomic force microscope an alternative method, based on holographic optical tweezers was developed within the work of this thesis. Optical tweezers (Fig. 2.3.) or optical traps are arguably the most sensitive single-molecule manipulation force measurement technique <sup>58</sup>. They can exert forces ranging from 0.2 pN to 100 pN on particles ranging in size from 0.5 nanometers to 5 microns while simultaneously measuring the three-dimensional displacement of the trapped

## A MFP 3D Base



## B MFP 3D Head

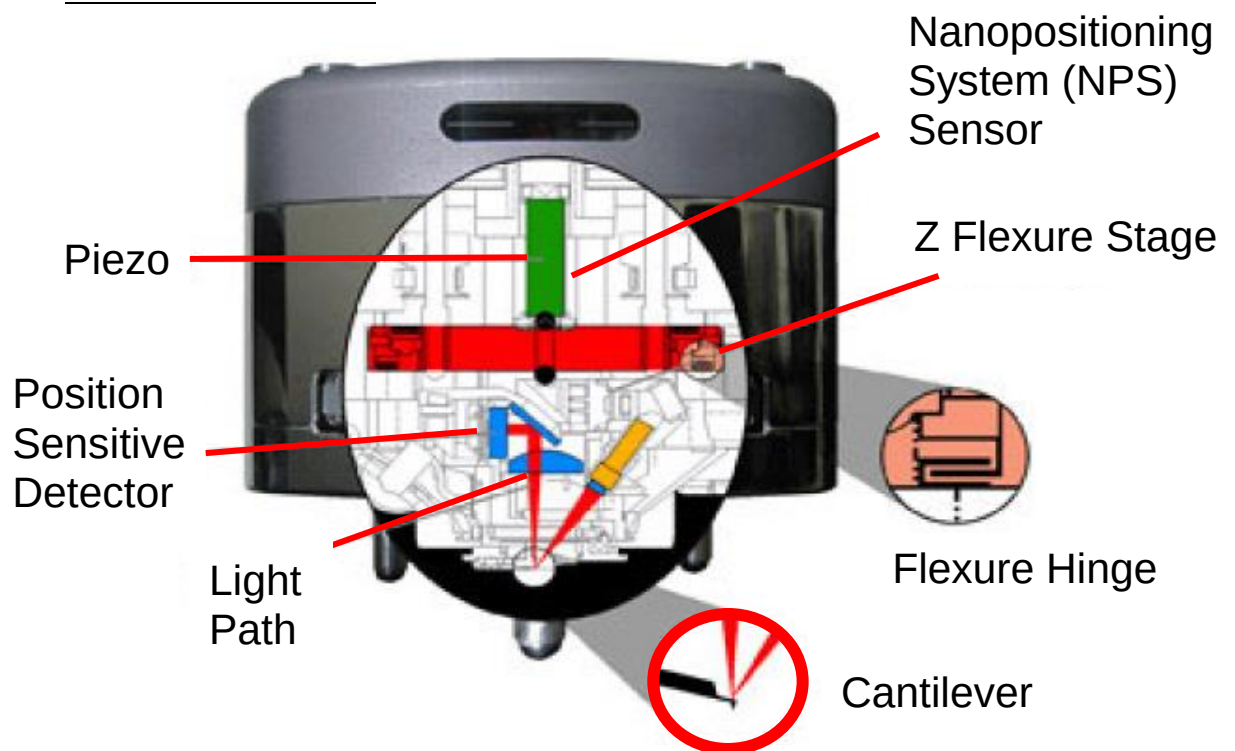


Figure 2.2. Basic components of the MFP-3D AFM used for force measurements<sup>87</sup>. (A) MFP3D AFM XY Base. The bottom view allows the observation of transparent and liquid samples. (B) The nanopositioning system (NPS) inside the MFP3D head enables precise tracking of the cantilever Z axis position. This allows the accurate measurement of rupture forces.

particle with sub-nanometer accuracy and sub- millisecond time resolution <sup>58</sup>, <sup>88</sup>. These properties make them extremely well suited for the measurement of force and motion <sup>62</sup>. Since their first demonstration in 1986 by Arthur Ashkin and his colleagues, OT have been utilized in a vast range of different physical and biological applications <sup>89-94</sup>. The ability to pick up and move biological molecules and cells without causing damage has expanded the research of mechanical aspects of biology.

In its most basic configuration (e.g. Fig.2.3.A) an optical trap is generated by focusing a single laser beam with a high quality microscope lens onto a specific position within a specimen plane under investigation <sup>95</sup>. The particle of interest is then captured in the centre of the laser beam focus by a force created by the light gradient and scattering processes, which both result from the interaction of the particle with light. Since their invention optical tweezers as instruments however have rapidly progressed from simple tools to manipulate micrometer sized objects into sophisticated analytical devices under computer control that can make reliable force, displacement and micro-rheology measurements with a high degree of precision and accuracy <sup>88, 96</sup>. As shown in Figure 2.3. optical traps can be considered a manifestation of the phenomenon that light is able to exert forces onto matter. Light waves coming from a laser create a pressure impact due to photons colliding with a surface. These waves also possess a momentum. The pressure and the momentum are both directed normal to the waves of light <sup>97</sup>. Once the light reaches and enters a dielectric particle the direction in which the light rays travel is altered due to the processes of reflection and refraction at the particle surface which causes the momentum of the light to change.

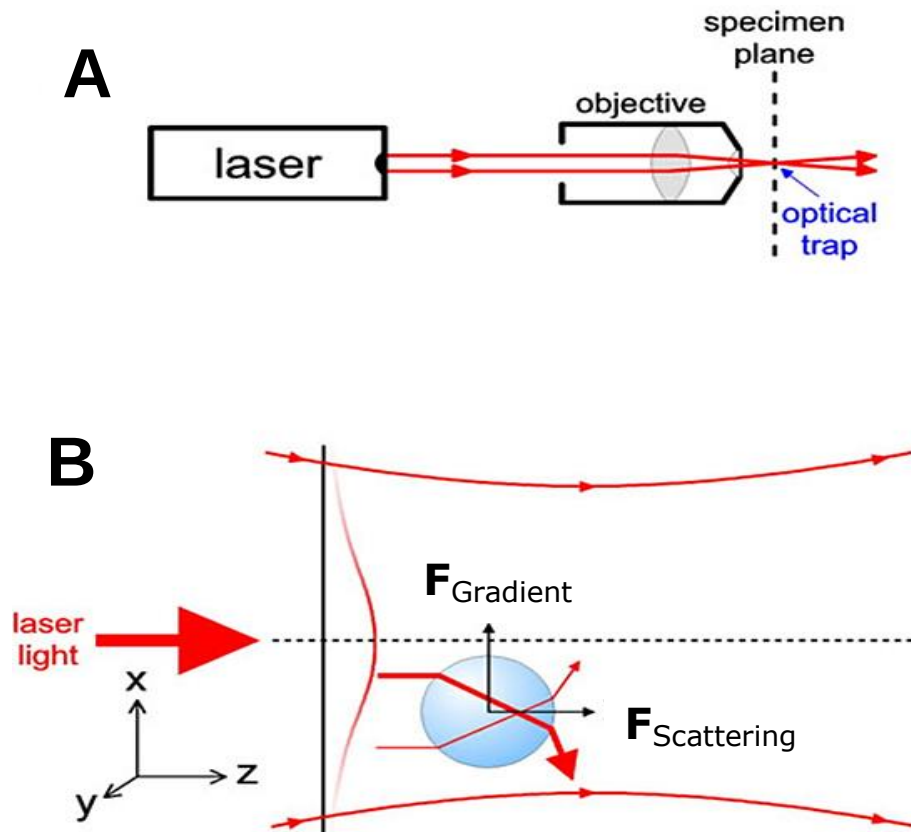


**Figure 2.3. Basic concept of optical tweezers.**

(A) A laser beam is focused on a specific location in the specimen plane where the optical trap is generated. Using only the refraction of light particles can be trapped and moved at will.

(B) As the light reaches the particle it is deflected at its surface. This creates a momentum and directional change of the light beam which combined with the existing field intensity gradient inside the trap has the capability to capture the particle <sup>95</sup>.

Photons coming from the laser that collide with the particle also create a scattering force (in the direction of propagation of the light), while a gradient in the field intensity produces a gradient force (directed towards the region of most intense light). Both of these forces are sufficient to trap the particle of interest firmly inside the focal plane<sup>98</sup>. According to Newton's third law, the conservation of momentum, when the light hits a particle with its full momentum the particle must undergo an equal and opposite momentum change. This momentum change creates a force that has the capability to trap the particle inside the laser focus.

The majority of optical traps used for laser OT employ the intensity profile of the Gaussian laser beam, which means that the light at the centre of the beam is much brighter than the light at the edges. In reality, the bead is constantly moving with Brownian motion and in the event the particle should happen to leave the centre of the optical trap a restoring force is created that is proportional to the displacement of the particle relative to the trap centre and will pull the particle back to the middle of the trap. This is due to the fact that the central beams possess a higher intensity and apply a larger momentum change towards the particle at the centre of the trap, compared to the external beams bearing a lower intensity, which employ a smaller momentum change at a distance to the trap centre<sup>95</sup>.

In most experimental applications OT start out as commercially available optical microscopes, which are then customized to suit the task at hand by adding extensive modifications to the original set up. While early optical tweezers set ups were commonly single-trap instruments, more recent versions frequently utilize time sharing or diffractive splitting of the Gaussian

laser beam to generate multiple optical traps in the focal plane e.g. using acousto-optic deflectors or diffractive optical elements respectively<sup>99</sup>.

Because in this case the optical traps are produced from a pattern of interference produced by a split coherent laser beam this set up of optical tweezers is also referred to as holographic optical tweezers (HOT).

In order to minimize the risk of radiation related damage to biological samples and to accomplish a high trap stiffness, Nd:YAG based high power infrared lasers at a wavelength of 1064 nm are commonly employed in biological applications of the optical tweezers. The manipulation of cells with infrared laser beams has been shown not to damage cells, even if the levels of light intensity are high e.g. 80 mW<sup>100</sup>. In summary: Optical trapping allows the application of forces in the femto-Newton range and the measurement of displacements in the nm regime for dielectric objects that range in size from 10 nm to over 100  $\mu\text{m}$ , making it a well suited instrument in the biological sciences for research ranging from the single-cell down to the single-molecule level<sup>88</sup>.

#### **2.4.1. Holographic Optical tweezers (HOT) setup**

Within the HOT set up (developed and built by the Padgett group, University of Glasgow), trapping is accomplished by using a diode pumped solid state laser (Laser Quantum, VentusIR) which provides up to 3 W at 1064 nm<sup>96</sup>.

The laser beam is expanded to fill the aperture of a spatial light modulator (SLM) (BNS, 512 pixels) coupled into a customized microscope using a polarizing beam splitter.

The spatial light modulator consists out of a matrix of liquid crystal pixels which are square and arranged in an XY pattern. The SLM is used to modulate the laser beam to create a 3D array or hologram of multiple optical traps. This allows the manipulation of several traps independently in all three dimensions with high accuracy, which is an advantage over conventional OT where only one bead can be trapped at one time <sup>102, 103</sup>.

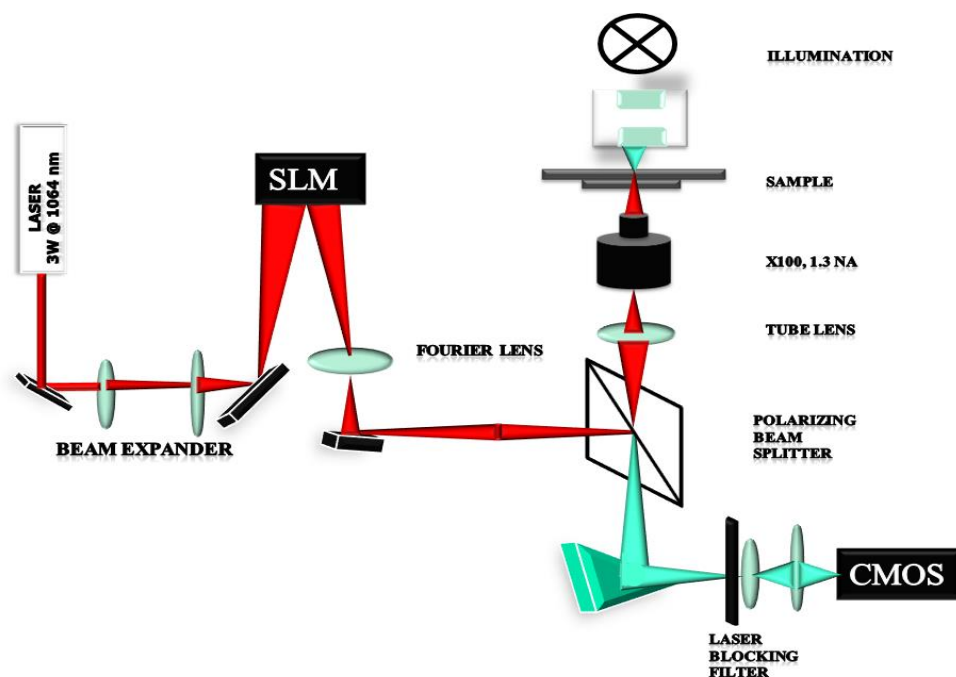


**Figure 2.4. Components of holographic optical tweezers (HOT) <sup>101</sup>. Trapping was achieved using a diode pumped solid state laser. The laser was expanded to fill the aperture of a spatial light modulator (SLM) which allowed multiple optical traps to be created, coupled into a custom microscope using a polarising beamsplitter. To acquire images of the motion of particles a complementary metal-oxide semiconductor (CMOS) camera was used which was connected to a desktop computer. A motorised microscope stage allowed accurate control of the sample slide position. The complete optical system was mounted on an air damped optical table in order to isolate the system from environmental sources of noise.**

The zero-order diffracted beam, in the center of the SLM, experiences no wave front modification because it is directly reflected off the SLM. The wave front of the first and higher-order diffracted laser beam (towards the edges of the SLM) however, are modified to match the desired amplitude, frequency and phase shift. This means that the trap characteristics will vary according to the position of the diffracted light emerging from the SLM and, must be characterized before force measurements can be carried out. An objective lens, 100x1.3 NA, (Zeiss, Plan-Neofluor) is used to both focus the trapping beams and to image the resulting motion of the particles, acquired using a complementary metal-oxide semiconductor (CMOS) camera (Prosilica) connected to a desktop computer. The particle tracking software, written in LabView (National Instruments), facilitates measurement of particle positions up to a few kHz for a reduced field of view of the camera. A motorized microscope stage (ASI, MS-2000) enables accurate control of the sample slide position and provides a means of dragging a trapped particle through the surrounding fluid. The complete optical system is mounted on an air damped optical table (Newport) in order to isolate the system from environmental sources of noise.

## **2.5. Dynamic force spectroscopy (DFS) - Exploring the kinetics of receptor-ligand interactions**

After the establishment of SMFS as a tool to measure forces in ligand-receptor interactions in the early 1990's (as described in Section 2.2. above)<sup>65-73</sup>, it was discovered later during that same decade that an unbinding force measured between receptors and ligands at constant pulling velocity

represents only a single point in a continuous spectrum of bond strengths <sup>104</sup> (Fig.2.5.A). These assumptions were based on different theories and experiments that suggested that bond rupture forces of a molecular bond depend on the loading rate of the force that is applied to that bond <sup>105-107</sup>. The loading rate is defined as the rate at which the load, that is the force  $F$ , is applied to a molecular bond, which can be derived from the product of the pulling speed and the effective spring constant of the force probe used <sup>60</sup>. The association and dissociation of any molecular bond is a stochastic process, that is influenced by many different factors such as random thermal fluctuations that drive the movement of molecules in a fluid, as well as collisions of molecules with other fast-moving molecules as described by Brownian motion <sup>57</sup>. This means that the rupture force of the same interaction measured multiple times will be distributed over a wide range of different magnitudes. Therefore a single force measurement is insufficient to characterize a molecular bond formed between two proteins. In order to characterize a molecular interaction therefore, the most probable rupture force (mode force) needs to be determined at a variety of loading rates <sup>63</sup>.

The study of the rupture force dependence on the loading rate of a biomolecular interaction is known as dynamic force spectroscopy (DFS) <sup>1</sup>. To obtain a dynamic force spectrum one typically obtains force measurements at different probe movement speeds, and the most frequent force (modal force) at each of these speeds is determined and plotted as a function of the loading rate on a semi logarithmic plot (see Fig 2.5.B). Again, the loading rate ( $\text{pN s}^{-1}$ ) is defined as the stiffness of the system ( $k$ ) multiplied by the probe speed <sup>60</sup>.

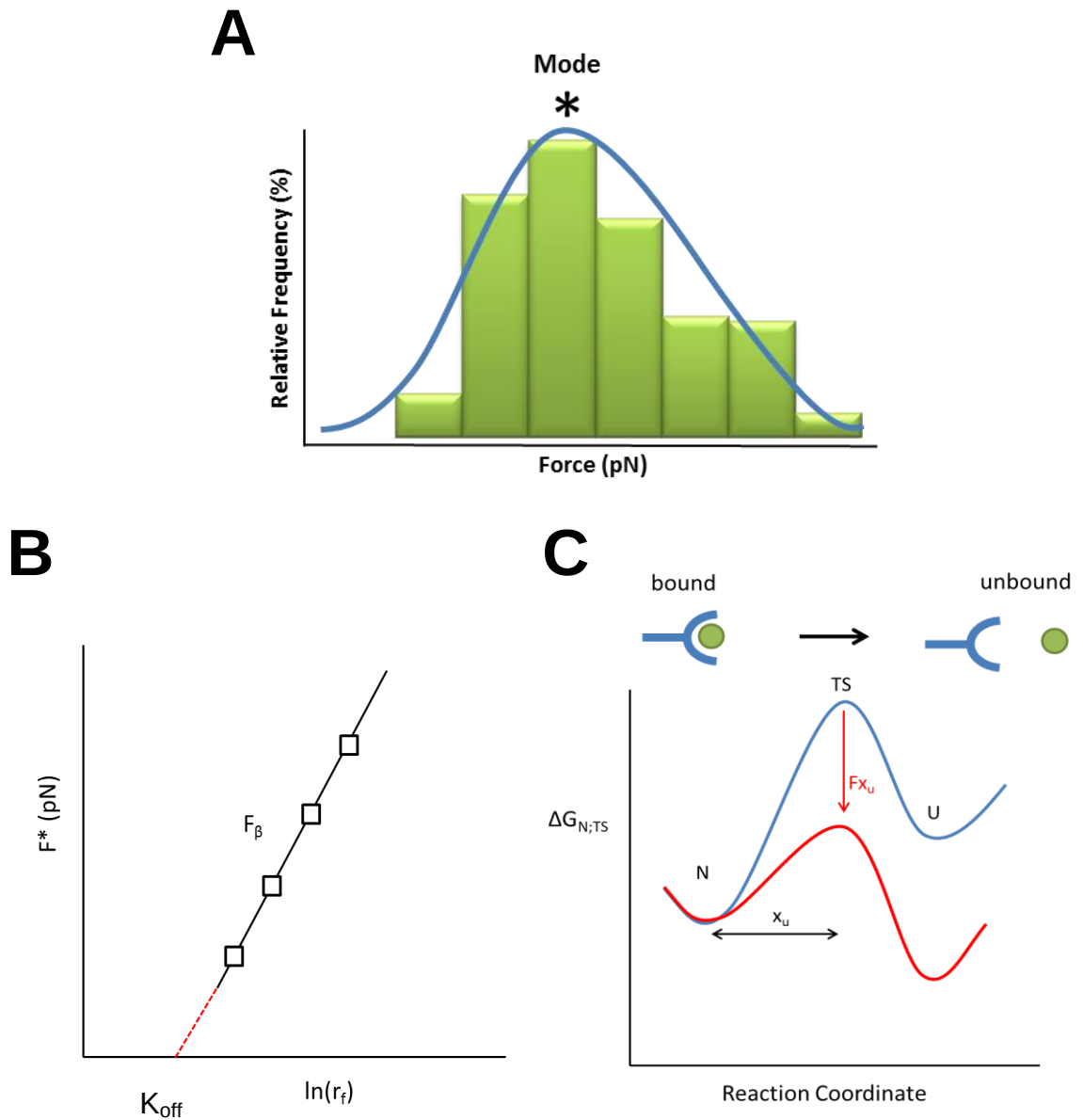


**Figure 2.5. Principles of dynamic force spectroscopy (DFS) <sup>108</sup>.** (A) Histogram showing the distribution of all rupture forces recorded in a series of force measurements recorded at one particular AFM tip speed. From this distribution the force mode can be determined. (B) Rupture force modes as a function of the loading rate. By extrapolation of the line the unstressed dissociation rate  $K_{off}$  can be determined. (C) Energy landscape of a receptor ligand interaction showing the bound state “N” the transition state “TS” and the unbound state “U”. An applied force “F” lowers the energy required to overcome the “TS”(red line) and reach “U”. The change in energy is given by the product of the force “F” and the distance from the bound state  $F(x_u)$ .

For biomolecular interactions the modal rupture force usually increases linearly as a function of the natural logarithm of the loading rate <sup>60</sup>. By extrapolation of the line connecting the measured force modes, the thermal force scale  $F_\beta$  and the unfolding rate at 0 pN force ( $k_{off}$ ) can be determined (Fig.2.5.B). The line slope corresponds to a given energy barrier ( $\Delta E$ ) and its distance ( $x_u$ ) from the bound state in the energy landscape of the bond. The energy landscape refers to a plot of all spatial positions of interacting proteins from bound to unbound states and their corresponding energy levels in Gibbs free energy (Fig.2.5.C). Each change in the slope represents a different energy barrier along the dissociation pathway <sup>106</sup>. The slope of the line obtained in the dynamic force spectra is given by

$$F_\beta = \frac{k_B T}{x_u}$$

Equation 2.2.

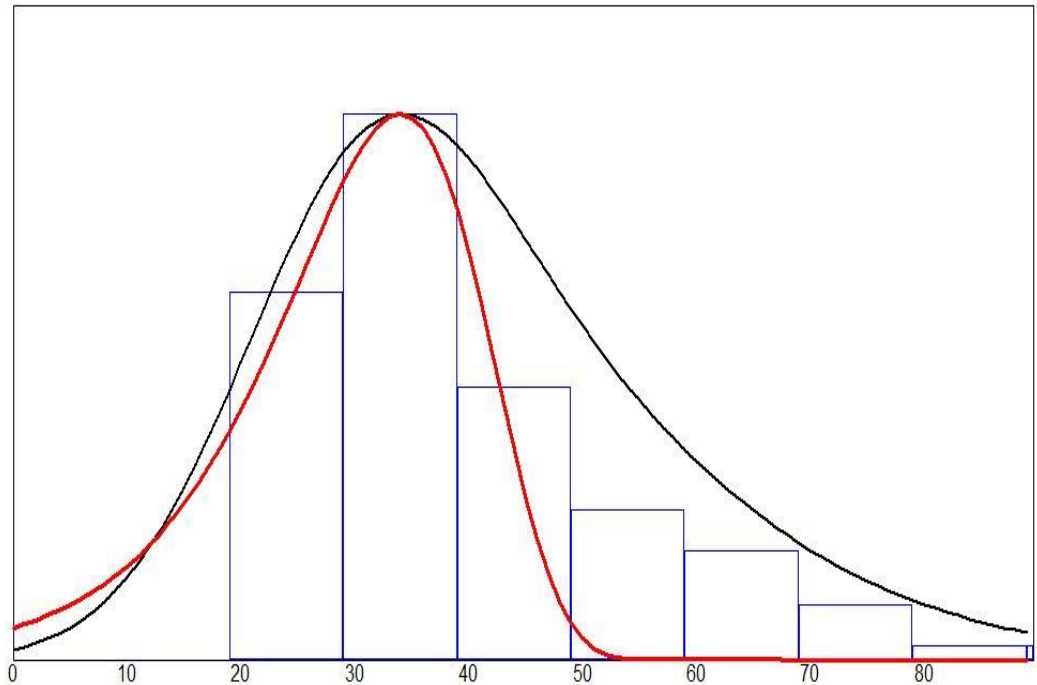
Where  $F_\beta$  is the thermal force scale,  $k_B$  is the Boltzman constant,  $T$  is the temperature and  $x_u$  is the distance between bound state and unbound state.

The relationship between loading rate and rupture force allows for an approximation of important information such as affinity, rate constants, and existing energy barriers in ligand-receptor interactions <sup>109</sup>.

For the work of this thesis, in order to recreate the probability distribution of measured rupture force magnitudes and to determine the most frequently occurring rupture force (mode force) the in house developed software FDist was employed <sup>63, 109, 110</sup>. Fdist. Is a program that was developed by Prof.

Phillip M. Williams (University of Nottingham). With Fdist, the probability distribution of bond rupture forces is created by drawing each rupture force measurement as a discrete measurement from a Gaussian distribution the width of which is set equal to the thermal noise level and force resolution of the force probe used (in our experiments the AFM cantilever with 10 pN). A cumulative distribution is created by summing up the Gaussian distribution of every single rupture force measurement. For this cumulative distribution the mode force, as peak of the cumulative distribution, is determined. The standard error of the mode was estimated using a Bootstrap method. Briefly, having determined the mode as the maximum in the cumulative distribution,  $1/e$  (approximately 37%) of the data was replaced with replicates and the mode recalculated. This resampling was repeated 100 times and the standard deviation of the modal values determined <sup>111</sup>. Figure 2.6.A shows an example distribution for a ligand-receptor interaction determined with Fdist (the example shown was taken from a force measurement of two interacting E-cadherin ectodomains). The predicted force distribution for the single bond interaction was determined from the modal force and approximate force scale (determined from the curvature of the distribution at the modal force value), and was shown with (red line) and without (black line) the outliers. After determining the mode with Fdist, the specific rupture force values were plotted as histograms (Fig.2.6.B) in Microsoft Excel showing the frequency which each force magnitude occurred with bin sizes equal to the width of the Gaussian chosen for each individual force measurement (10 pN) and with an offset so that the largest bin surrounds the modal value of the cumulative distribution.

**A**



**B**

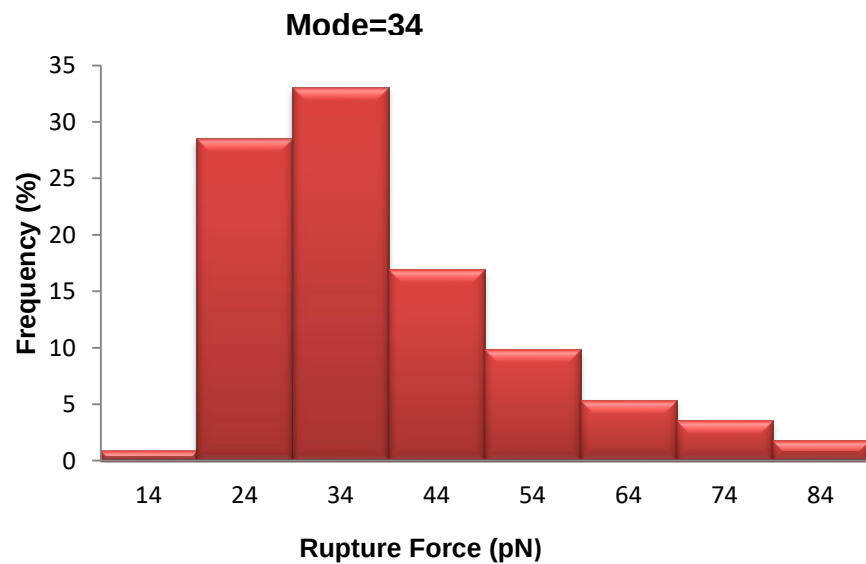


**Figure 2.6.** Statistical analysis of the measured rupture forces measured between E-cadherin functionalized AFM tips and silicon surfaces at  $1 \mu\text{m s}^{-1}$ . (A) The specific forces are fitted using the home developed program Fdist<sup>63, 109</sup>. (B) The data is then plotted as a histogram with a bin size of 10 pN to show the distribution of the specific rupture force magnitudes centred around the mode force.

## 2.6. Dynamic Force Spectroscopy (DFS) - A useful theory to study protein interactions

In order to illustrate the usefulness of DFS, especially when combined with other analytic methods, to study internal and external protein interactions, an early (1999) as well as a recent example (2015) will be briefly summarized here. First, in a landmark study by Merkel *et al.*, in 1999, the bio membrane force probe (BFP) was used to measure the bond strength spectra for the bonds between streptavidin or avidin and biotin, for the first time, over six orders of magnitude in loading rate<sup>104</sup>. The BFP tip (a glass microbead of  $\sim 2$   $\mu\text{m}$  diameter) was functionalized with a small amount of streptavidin or avidin and brought into and out of contact with biotin on the sample surface (see Figure 2.7.A). The most frequent rupture forces (the force modes (see Figure 2.7.B)) were plotted against the loading rate at which they were measured (see Figure 2.7.C) and a linear dependence of the rupture force on the loading rate could be observed. The bond survival time diminished from about 1 min to 0.001 s with increasing loading rate while, bond strengths increased from about 5 pN to 170 pN. This suggested that these bonds appear strong or weak depending on how fast they are loaded. The slope of each of these linear trends of the force spectra was interpreted to be a sharp energy barrier at a fixed location along the unbinding pathway in the energy landscape (see Figure 2.5.C). This study showed that in order to study molecular interactions by measuring forces with force probes, force measurements had to be performed over a wide range of loading rates in order to accurately describe the molecular interaction under investigation.

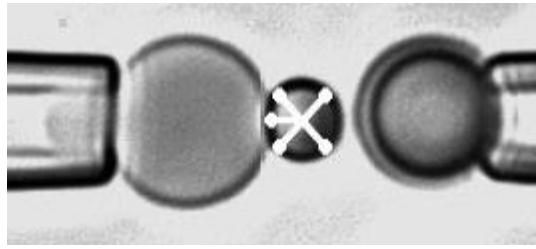
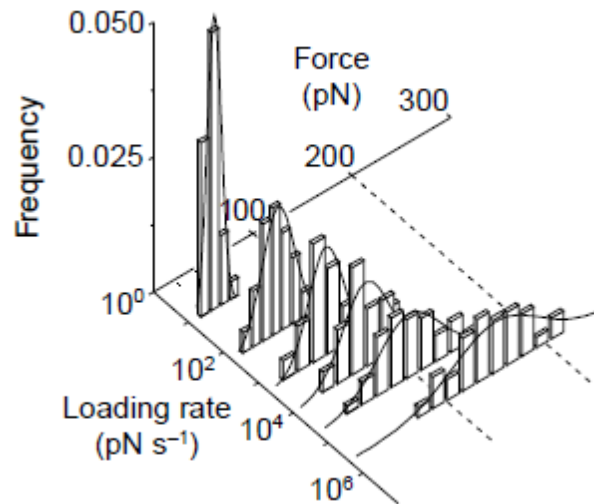
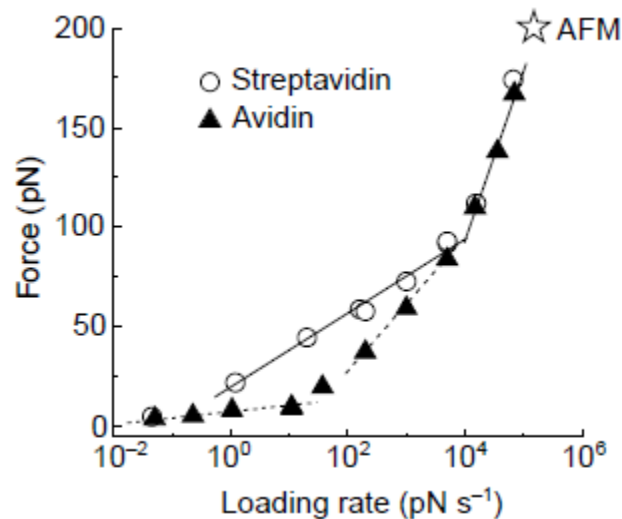
**A****B****C**

Figure 2.7.DFS example I <sup>104</sup>. (A) The spring in the biomembrane force probe BFP is a pressurized red blood cell (on the left). The BFP tip is a glass micro-bead of that was chemically glued to the membrane of the red blood cell and was controlled by micropipette suction. The bead is then brought into and out of contact with a second bead surface (on the right) functionalized with biotin for force measurements. (B) Force histograms from tests of single biotin-streptavidin bonds showed a shift in peak location and increase in width with increase in loading rate. Gaussian fits were used to determine the most frequent rupture force (modal rupture force). (C) Dynamic strength spectra for streptavidin-biotin (circles) and avidin-biotin (triangles) bonds. Each change in slope was interpreted as a different energy barrier on the unfolding pathway of the ligand receptor interaction.

In the second example Stagner et. al., combined in a recent study (2015) enzyme-linked immunosorbent assay (ELISA) with molecular dynamics simulation (MD) and optical tweezers-assisted dynamic force spectroscopy (DFS) <sup>112</sup>. These researchers aimed to identify for the first time the binding epitope of a phosphorylation specific monoclonal antibody on the Alzheimer's disease relevant peptide tau, on the level of single amino acids. Seven specific tau isoforms were synthesized by replacing binding relevant amino acids by a neutral alanine. First ELISA was used by measuring the maximum optical density (OD-100) of serial dilutions for the binding of the specific antibody to the original peptide and then to the seven alanine-scan derivatives. Based on the serial dilution curves from the ELISA measurements, the affinity constant  $K_a$ , could be determined which is one of the most important parameters for determining the strength of an antibody–antigen interaction <sup>113</sup>. After scanning the antibody binding epitope with ELISA, they investigated the interactions between the antibody and the eight tau peptides using a single molecule based approach. For that purpose they used an optical tweezers (OT) based method in which they fixed a single peptide-coated bead on a custom-made micropipette using capillary forces. Subsequently, a second bead covered with the antibody was captured and placed in close proximity to the bead fixed on the pipette (Fig.2.7.A, position (1)). Using a custom-made LabView program, both particles were approached until a force of  $5 \text{ pN} \pm 2 \text{ pN}$  was reached (position (2)). After 1 s of contact they were retracted with a preset velocity. If a bond had formed, the bead in the laser trap was shifted out of its equilibrium position until the bond force was exceeded and

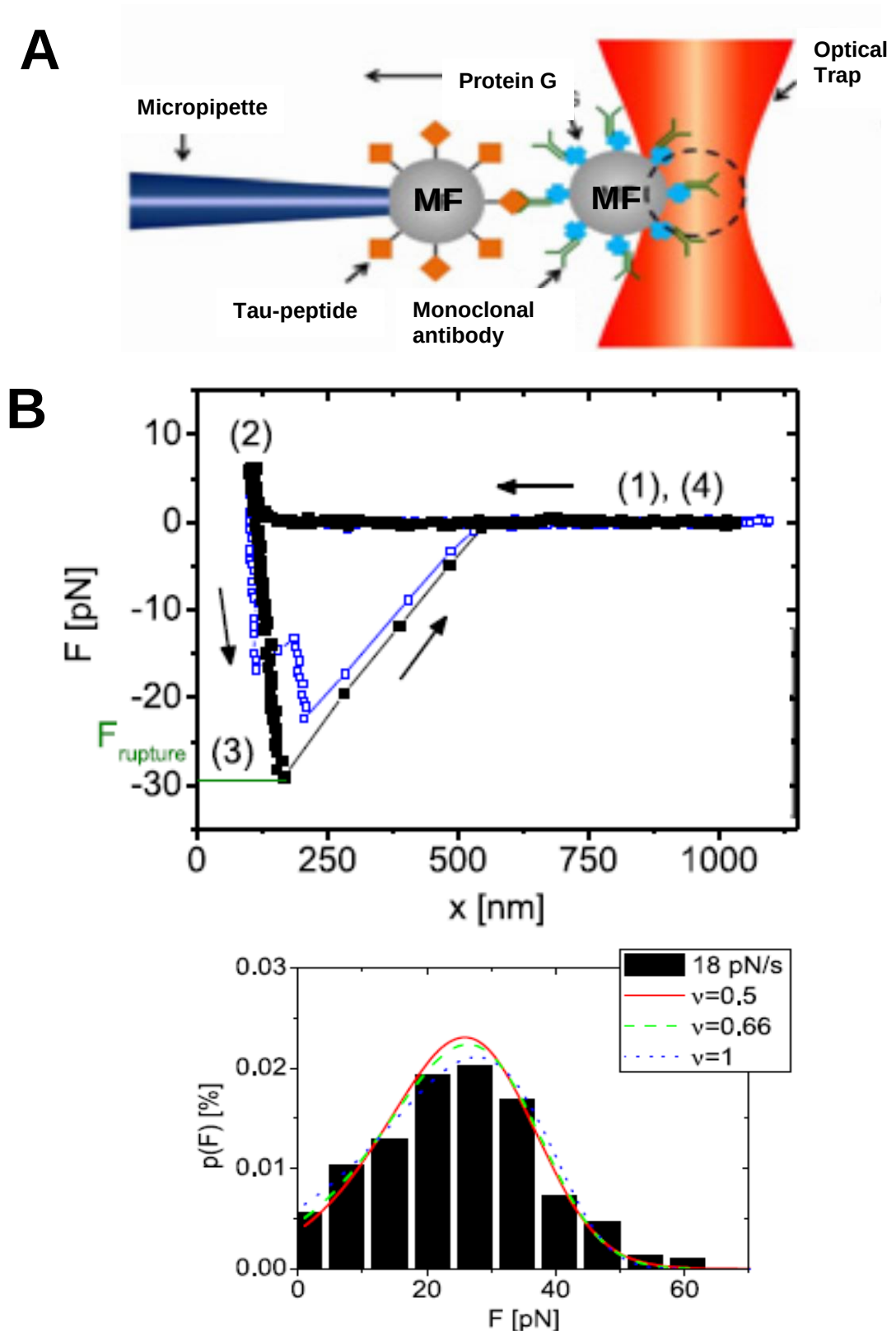


Figure 2.8. DFS example II <sup>112</sup>. (A) The tau peptide-coated bead is immobilized on a micropipette by capillary action. The second particle (coated with Protein G and the specific antibody) is held in the optical trap. (B) Example force-distance curve for a rupture of a single receptor-ligand bond (at a loading rate of 18.2 pN s<sup>-1</sup>). To establish a bond, the coated beads are brought closer together until a force of 5 pN  $\pm$  2 pN is reached (position (2)) and pulled apart with a pre-set velocity after 1 s of contact. As a result of the interaction between the peptide and the antibody, the particle in the optical trap is deflected from its equilibrium position until the bond ruptures (rupture force, position (3)). Afterwards, the system goes back into its initial state (positions (1),(4)). Force-distance curves containing multiple bond ruptures (open blue symbols) were not included in the data analysis. (C) Example rupture force histogram for the interaction between the antibody and the tau peptide.

the bond was ruptured (position (3)). Subsequently the particle jumped back into its initial position (position (4)). This procedure was repeated at different loading rates, which ranged from 18 pN to 99 pN/s for all receptor–ligand combinations. For each loading rate, between 1000 and 1500 force–distance curves were recorded, resulting in at least 200 rupture events. Based on the determined binding parameters such as kinetic rates (on- and off-rate) and the affinity constant  $K_a$ , Stagner *et al.* categorized the importance of each tested amino acid as essential, secondary and non-essential amino acids for antibody recognition, purely based on specific binding properties of the investigated antibody–peptide complex. To test whether the observed differences in binding parameters were caused by structural rearrangements upon amino acid substitution, the protein structures were simulated for each isoform using MD simulations. Two parameters were chosen to monitor these changes: the distance between the  $\alpha$ -carbons ( $d_{C-C}$ ) between the residue 231 and 235 (i.e. those which had been previously measured to be the binding epitope of the antibody) and the  $d_{E-E}$  along the peptide backbone. They found that the  $d_{C-C}$  was mostly conserved upon residue substitution. In contrast, the  $d_{E-E}$  changed strongly depending on the type of amino acid substitution which confirmed the observed amino acid classification.

In conclusion in the study by Stagner *et al.*, SMFS in combination with DFS and MD provided a model to study the mechanism by which specific antibodies recognize a disease related protein. The authors proposed that this antibody binding epitope may provide the basis for future development of highly selective antibodies for the diagnosis and treatment of neurodegenerative diseases such as Alzheimer's and Dementia and that the

information acquired would not have been obtainable by solution based methods.

These examples show how force SMFS and DFS measurements can provide unprecedented insights into the molecular mechanisms and selectivity of protein bonds. Protein structures are only one form of physical evidence for a molecular mechanisms and it is impossible to see bond kinetics, energies or adhesion bond strengths from just structural features alone <sup>18</sup>. Although visualization methods based on fluorescence probes allow the detection of single molecules under certain conditions these techniques are essentially passive observation methods and do not allow the activity of proteins to be studied <sup>4, 114, 115</sup>. Furthermore genetic perturbation methods such as clustered regularly interspaced short palindromic repeats (CRSIPR), single gene knockout and small interference RNA (siRNA) based techniques face many complications from adaptive mechanisms and off-target effects that obstruct the normal function of the cell in which the expression of a particular gene is silenced <sup>116</sup>. Models of the cadherin binding mechanism must thus be substantiated not only by structural information but also by quantitative biophysical measurements that are capable of testing proposed mechanisms. The aim of this thesis was to help close this knowledge gap by investigating how biophysical properties of cadherin bonds between stem cells change as they differentiate into specific tissue lineages.

# **Chapter III**

## **Cadherin interactions probed by AFM force measurements**

### **3.1. Introduction**

#### **3.1.1. Force measurement studies of cadherin interactions**

Given their importance in many mechanical aspects of cell adhesion and tissue maintenance it is perhaps not surprising that force spectroscopy methodologies have been used to advance the understanding of cadherin interactions. Initial force-related studies of cadherin binding behaviour focused primarily on the magnitude and distance dependence of bonds formed between full-length antiparallel interdigitated extracellular cadherin segments<sup>15, 117</sup>. Surface force apparatus (SFA) measurements of interacting C-cadherin ectodomains revealed multiple types of bonds formed between bound cadherin ectodomains<sup>117</sup>. When cadherin-coated surfaces were “pulled apart,” force maxima at multiple distances were observed. The strongest bonds formed between two fully interdigitated antiparallel proteins, whereas, when proteins were separated by a distance equal to the length of one subdomain, weaker, low affinity bonds formed corresponding to the EC1 subdomain of one C-cadherin binding to subunits EC1 through EC4 on the opposite C-cadherin ectodomain.

Similarly, more recent investigations using the biomembrane force probe (BFP) further characterized the different types of bonds that form between C-cadherin subdomains <sup>15</sup>. *Trans* bonded-cadherin subunits EC 1 and 2 (formed two weak short-lived bound states whereas the full ectodomain (EC1-5) displayed two long-lived states in addition to the fast lived bonds observed with antiparallel EC1-2 fragments. This supported a model in which the extracellular cadherin domains bind using a multistate binding mechanism. The differences in binding kinetics and binding strength determined for each bond type identified could be assigned directly to distinct domains on the modular architecture of cadherin ectodomains.

Baumgartner *et al.* were amongst the first to use AFM to characterize binding strength and binding kinetics of a full length (EC1-5) classical cadherin ectodomain at the single molecule level <sup>118</sup>. In their experiments they used vascular endothelial (VE)-cadherin, functionalized to an AFM cantilever and to the surface of SiOH plates to bring the cadherin ectodomains into and out of contact with one another. Furthermore, they allowed the tip to dwell on the surface for a defined amount of time (e.g. 0.1 s). They then retracted the tip at different speeds and obtained characteristic unbinding curves with distinct force jumps in the retraction curve of the AFM tip and attributed these to the bond rupture of bound cadherin molecules.

From this they generated frequency distributions of these rupture events which showed three distinct peaks with modal rupture forces of 41 pN, 75 pN and 120 pN (for a tip retraction speed of 800 nm s<sup>-1</sup>). These findings were found to be in agreement with similar AFM studies performed by Fichtner *et al.* who used full length (EC1-5) E-cadherin ectodomains at defined lateral

densities and found two distinct peaks at 35 pN and 60 pN, at a similar retraction speed ( $1 \mu\text{m s}^{-1}$ )<sup>12</sup>. In the AFM studies by Baumgartner and Fichtner, the tip retraction speeds used were varied, after a defined dwell time of the tip on the surface. It was found that the unbinding force measured, increased linearly with the natural logarithm of the loading rate. Fichter *et al.* also probed the bonds of the first two N-terminal E-cadherin subdomains (EC1-EC2) which included the calcium binding interdomain in between them with AFM and found a characteristic peak centred around 20 pN to 30 pN which du Roure *et al.* also identified in an independent AFM study focusing on the bond formation of a truncated version of the E-cadherin ectodomain<sup>119</sup>. This peak also corresponds to the first of three force peaks identified by Baumgartner *et al.* (41 pN) mentioned above.

The rupture force peaks identified by the groups of Baumgartner, Fichtner and du Roure strongly resembled those previously discovered by Sivasankar *et al.*<sup>117</sup> using the SFA and BFP, in that they could discriminate between weak types of bonds that form between a subset of the five subunits of the extracellular cadherin domain and stronger types of bonds that formed between two fully interdigitated cadherin ectodomains.

Zhang *et al.* attached E-cadherin ectodomain constructs either in the form of dimers or monomers to AFM tips and to glass coverslips to investigate the importance of *cis* dimerization in cadherin binding prior to *trans* interactions<sup>13</sup>. They found that both the dimer and the monomer had similar bond strengths ( $53 \text{ pN} \pm 27 \text{ pN}$ ,  $64 \text{ pN} \pm 27 \text{ pN}$ , respectively) but the dimer had a 3.4-fold higher binding probability compared to the monomer due to an increase in cadherin density.

Although the aforementioned studies could reveal the basic molecular mechanisms with which cadherins mediated cell adhesion, the way cadherins can drive cell differentiation and adhesion, especially in early development e.g. embryonic development using their physical capabilities, has not yet been fully elucidated. This chapter describes the first steps in addressing this issue. To investigate how the binding capabilities of cadherins mediate cell adhesion on pluripotent mES cells the extracellular domains of either E or N-cadherin were functionalized to the probe tip of an AFM cantilever and to the surface of silicon wafers. By bringing the functionalized AFM tip and silicon surface into contact with one another using AFM, specific interactions between cadherin ectodomains could be probed. This provided the basis for modelling cadherin mediated intercellular adhesion.

## **3.2. Materials and Methods**

### **3.2.1. Materials**

#### **3.2.1.1. Buffers used for AFM force measurements**

Force measurements using the AFM (Molecular Force Probe (MFP3D; Asylum Research) were carried out in a HEPES buffer based liquid environment (5 mM HEPES, 150 mM NaCl, adjusted with NaOH to pH 7.4) containing either 5 mM  $\text{CaCl}_2$  or 5 mM EGTA/ 5 mM EDTA. HEPES buffer, EDTA and EGTA reagents were purchased from Sigma–Aldrich (Dorset, UK). Ultrapure water for buffer preparation was obtained from a Maxima ELGA purification system (resistivity 18.2 M $\Omega$  cm).

### **3.2.1.2. The structure of the recombinant cadherin Fc chimera**

The E- and N-cadherin-Fc fusion protein was purchased from R & D Systems (Abingdon, OX14 3NB). These proteins were recombinantly produced in mouse myeloma cells. The structure of the E-cadherin construct used in force measurements is shown in Figure 3.1.A. The five subdomains of the extracellular domain of the cadherin were attached to the Fc region of either a human IgG (E-cadherin) or a Mouse IgG (N-cadherin)( Fig.3.1.B) <sup>120, 121</sup>.

In its wild type protein expression, Type I classical cadherins possess an intracellular domain which includes the catenin cytoskeletal complex with p120 catenin, alpha catenin, beta catenin and vinculin (see Chapter I, Section 1.2.) <sup>123</sup>. This intracellular domain is absent in the protein constructs (Fig.3.1.C), however, it has been demonstrated previously that the extracellular domain of cadherin by itself is able to induce and mediate cell attachment and differentiation <sup>10</sup>.

To ensure the correct orientation of the cadherin ectodomain when it is attached to AFM tips and silicon surfaces, the primary structure of the cadherin ectodomain as well as the attached Fc domain was analysed for its lysine content using the Uniprot protein online database <sup>123</sup>. Lysine is an amino acid that has an amine group in its side chain. By determining the lysine density in the E- and N-cadherin ectodomain and comparing it to the lysine density of the Fc domain of the constructs, it was possible to show that the Fc domains have a higher content of amine groups (10%) compared to that of the cadherin ectodomain part (4%) and were thus

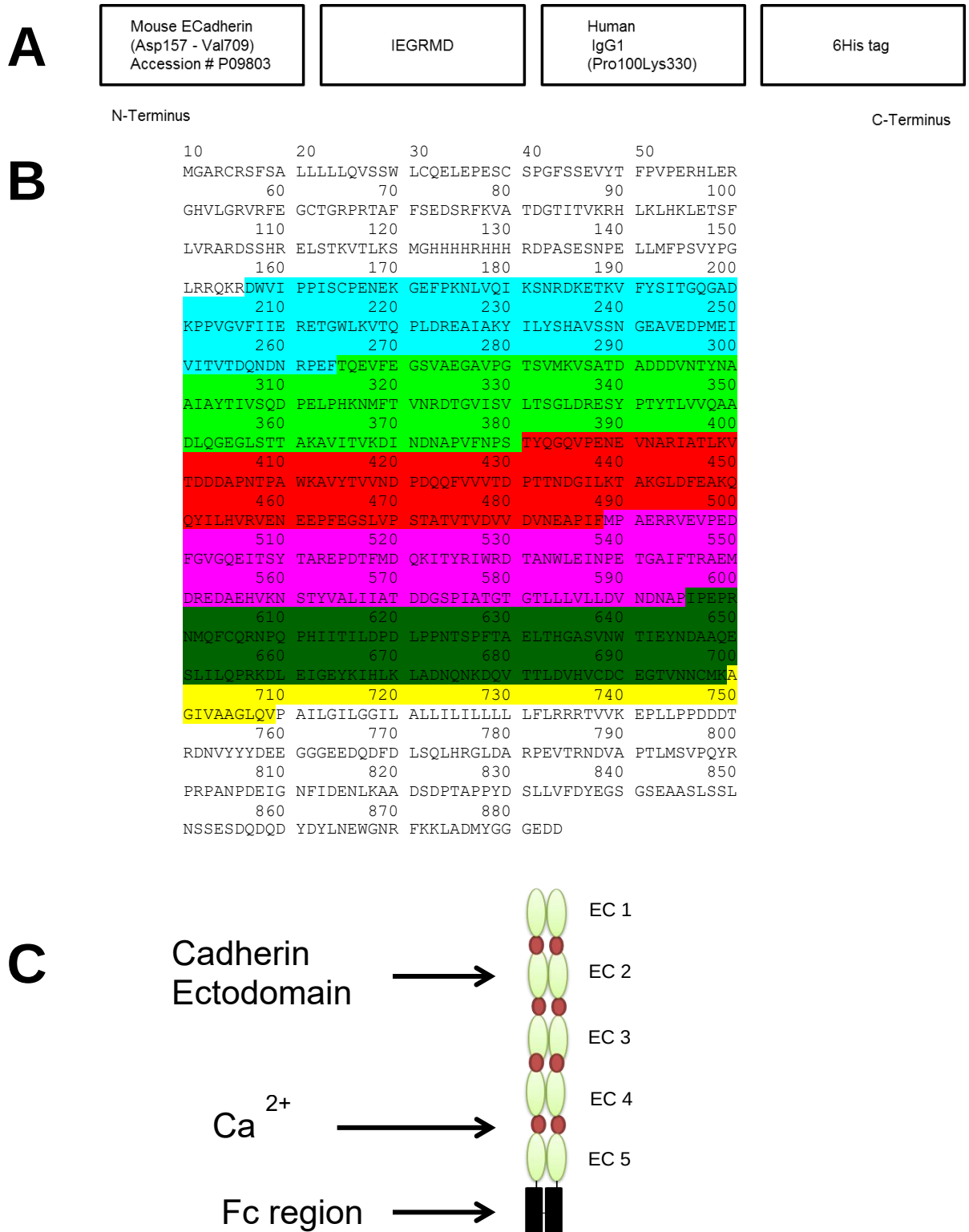


Figure 3.1. (A) Structure of E-cadherin constructs <sup>120</sup>. (B) Protein primary structure of mouse E-cadherin <sup>123</sup>. The highlighted areas show the extracellular domains (EC's) of E-cadherin that are attached to the Fc region of a human IgG1. The extracellular domain is further divided into 5 subunits (EC's) shown here as turquoise (EC1) green (EC2) red (EC3) purple (EC4) and dark green (EC5). The yellow region indicates a remainder of the membrane spanning part of cadherin that is used to attach the ectodomain to the Fc part of the construct but is not part of the extracellular subdomains. (C) Schematic of E-cadherin constructs used for dynamic force spectroscopy. The extracellular structure of classical cadherins contains five subdomains of the cadherin ectodomain connected by flexible hinge regions.  $\text{Ca}^{2+}$  ions bind to each hinge preventing it from flexing. As a result the extracellular region forms a rigid, rod like structure. Each cadherin is attached to one Fc region of the human IgG which makes E-cadherin form a dimeric structure due to the disulphide bonds connecting the Fc regions.

more likely to bind to the AMAS cross linker because of its affinity for amine (E-cadherin (Ectodomain 4%; Fc domain 9%); N-cadherin (Ectodomain 4%; Fc domain 8%)). Furthermore, because the same antibody was used for the immunostaining experiments presented in Chapter IV as well as for the blocking experiments of the AFM force measurements in this chapter and Chapter V, it was assumed that the cadherin construct would have the correct configuration otherwise the E- and N-cadherin specific antibodies would not have recognized the extracellular cadherin fragment.

### **3.2.2. Methods**

#### **3.2.2.1. Functionalization of silicon surfaces and AFM tips with E & N- cadherin ectodomains**

E or N-cadherin were functionalized to the probe tip of an AFM cantilever and to the surface of silicon wafers by using the surface vapor silanization bio conjugation strategy developed in collaboration with Dr. Rhiannon Creasy based on a preexisting functionalization protocol by Prof. Peter Hinterdorfer at the University of Linz, Austria <sup>124, 125</sup>. Figure 3.2. illustrates the five essential steps in the functionalization process: First, AFM silicon nitride tips (MLCT Bruker) and cleaved silicon wafers (Rockwood Electronic Materials) were cleaned by bathing them in acetone and ethanol, and placing them in an UV-cleaner (Bioforce Nanosciences) for 30 minutes (Step 1). Then the AFM tips and silicon wafers were functionalized with thiol (SH) groups by placing them inside an airtight desiccator filled with argon and two small trays, one with 30  $\mu$ L of (3-mercaptopropyl)trimethoxysilane (Sigma-

Aldrich) and one with 10  $\mu\text{L}$  of trimethylamine (Sigma Aldrich) for 2 hours, a process known as vapour silanization (Step 2) <sup>126</sup>. The tips and wafers were then rinsed again using acetone and then ethanol, and functionalized with the AMAS (N- $\alpha$ -maleimidoacet-oxysuccinimide ester) cross linking agent (Thermo Scientific) (Step 3). AMAS is an amine-to-sulfhydryl crosslinker that contains NHS-ester and maleimide reactive groups at opposite ends of a very short spacer arm (4.4 Å).

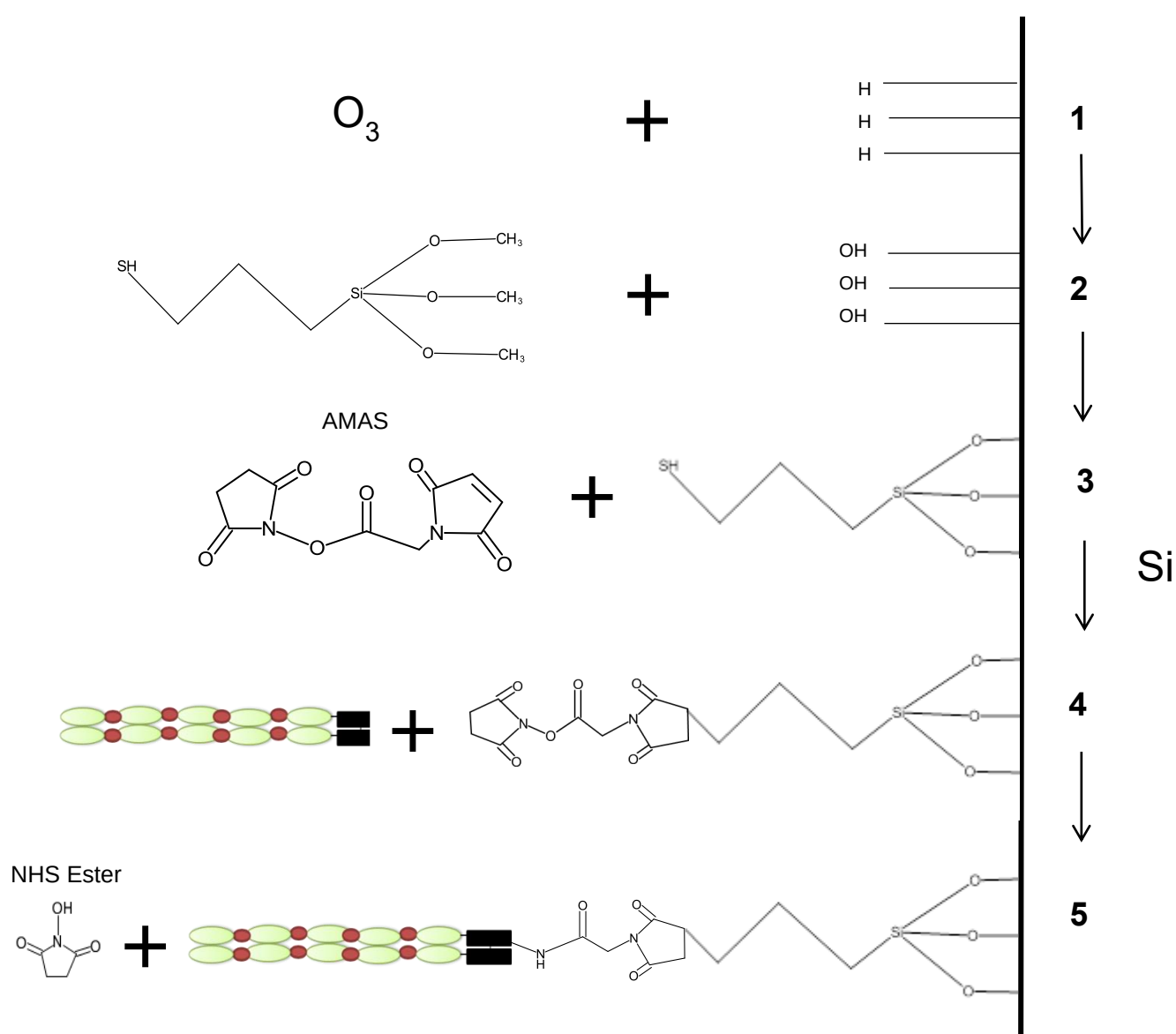


**Figure 3.2. Immobilization steps for E & N-cadherin constructs on AFM tips and sample surfaces. (1) UV cleaning using Ozone, (2) Attachment of (3-mercaptopropyl)trimethoxysilane, (3) Formation of thioether bonds between maleimide and surface bound thiol ( $\text{SH}$ ) groups, (4 and 5) Formation of amide bonds between the surface bound maleimide and amine groups on the cadherin fusion protein construct.**

The AFM tips and silicon surfaces were bathed in a solution of AMAS dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich) for three hours. Then in the final stage of the functionalization process the NHS group of AMAS was attached to NH<sub>2</sub> groups within the Fc part or the recombinant cadherin ectodomain (R &D Systems) (Step 4 and 5). To this end E – cadherin and N-cadherin constructs were reconstituted in 500 µL sterile PBS at a concentration of 100 µg/mL and AFM tips and wafers and were immersed into the cadherin solutions and left to incubate at 4 °C for at least 12 hours.

### **3.2.2.2. AFM measurements of E-cadherin interactions**

E-cadherin constructs were functionalized onto AFM tips and silicon wafers and force measurements were recorded in HEPES buffer. The silicon wafers were placed on the bottom stage of the MFP and the cantilever positioned over the wafer. The wafer was approached with the AFM tip to establish contact. Upon retraction, tip-surface interactions caused deflections of the cantilever, which were translated into forces as described in Chapter II, Section 2.3. Force curves were recorded using the AFM at four different tip approach and retraction speeds (1 µms<sup>-1</sup>, 500 nms<sup>-1</sup>, 100 nms<sup>-1</sup>, 50 nms<sup>-1</sup>) over a distance of 500 nm from the sample surface. The dwell time was set to 0 seconds to minimize the occurrence of multiple bond events (See Section 3.3.4 below). The force of approach (i.e. impingement force) was limited to a maximum of 100 pN. Rupture forces were determined directly by the height of the bond rupture force jumps from tip retraction curves of the adhesion force as function of distance using the Igor Pro software. In each

experiment, the tip - surface interaction was measured 500 times at three different positions on the silicon wafer for each tip speed and the modal bond rupture force was determined using the in-house developed software Fdist<sup>63, 109</sup> (See Chapter II, Section 2.5.). As a control, at the end of each experiment, an E or N-cadherin specific antibody (Goat anti-mouse E-Cadherin (R&D), Sheep anti Mouse N-cadherin, (R&D)) or EGTA or EDTA was added to the HEPES buffer. For each AFM tip the spring constant (k) was determined at the end of each experiment (See Chapter II, Section 2.3.).

### **3.3. Results and Discussion**

#### **3.3.1. Acquisition of force measurements –A worked example**

In order to study the interaction of cadherin ectodomains attached to AFM tips and silicon surfaces, a method was developed in which cadherin bond formations could be studied under near physiological conditions. This procedure will be briefly outlined in this section using a worked example in order to illustrate and explain the experimental procedure applied to gain the data of the single molecule force spectroscopy (SMFS) experiments.

First, an E- or N-cadherin functionalized AFM cantilever was inserted into the AFM head. The AFM head was then mounted on top of the glass slide on which the functionalized silicon surface was immobilized covered with a drop of HEPES buffer. This created a liquid chamber that allowed force measurements to be done in a liquid physiological environment. The tip-

surface interactions were then measured and recorded 500 times, for three different AFM tips, at three different positions on the functionalized silicon surface over a distance of 500 nm from the silicon surface (Fig.3.3. to 3.5.). The force curves recorded for each speed were then analysed by going through all 1500 force curves for each tip speed individually on the Asylum Igor Pro software (see Chapter II, Section 2.3.) and identifying, counting and measuring the magnitude of specific rupture events that occurred during the retraction of the AFM tip from the silicon surface. Only about 10% to 20% of all force curves showed specific adhesion (Fig.3.3.). The rest of the force curves showed unspecific adhesion (Fig.3.4.) or no adhesion events at all (Figure 3.5.). Specific adhesion curves showed a delayed, nonlinear trend after the point of contact in the retraction curve. This nonlinear trend was interpreted to reflect the stretching of the attached protein and linker molecules with increasing pulling force. Curves showing specific cadherin unbinding events were furthermore identified as those retraction curves that occurred within the first 150 nm away from the sample surface. Curves showing unspecific adhesion, on the other hand, were identified by force jumps that had a linear appearance prior to bond rupture (Fig.3.4.A) or occurred at zero distance from the surface (Fig.3.4.B). Such curves were attributed to interactions between the underlying tip and sample surfaces and were eliminated from subsequent analysis. Curves with adhesion events that ruptured at distances of 150 nm or more were also not selected for analysis as their origin could not easily be ascribed considering the molecular dimensions of the silane chemistry (0.6 nm)<sup>126</sup>, the spacer molecule (0.44 nm)<sup>127</sup> and the completely unfolded cadherin ectodomain (270 nm)<sup>120</sup>

(Fig.3.4.C). The measurement and analysis of rupture forces was repeated for four different tip speeds for each AFM tip ( $1 \mu\text{m s}^{-1}$ ,  $500 \text{ nm s}^{-1}$ ,  $100 \text{ nm s}^{-1}$ ,  $50 \text{ nm s}^{-1}$ ) in order to generate data for a dynamic force spectrum. Datasets were analysed by manually screening for adhesion events,

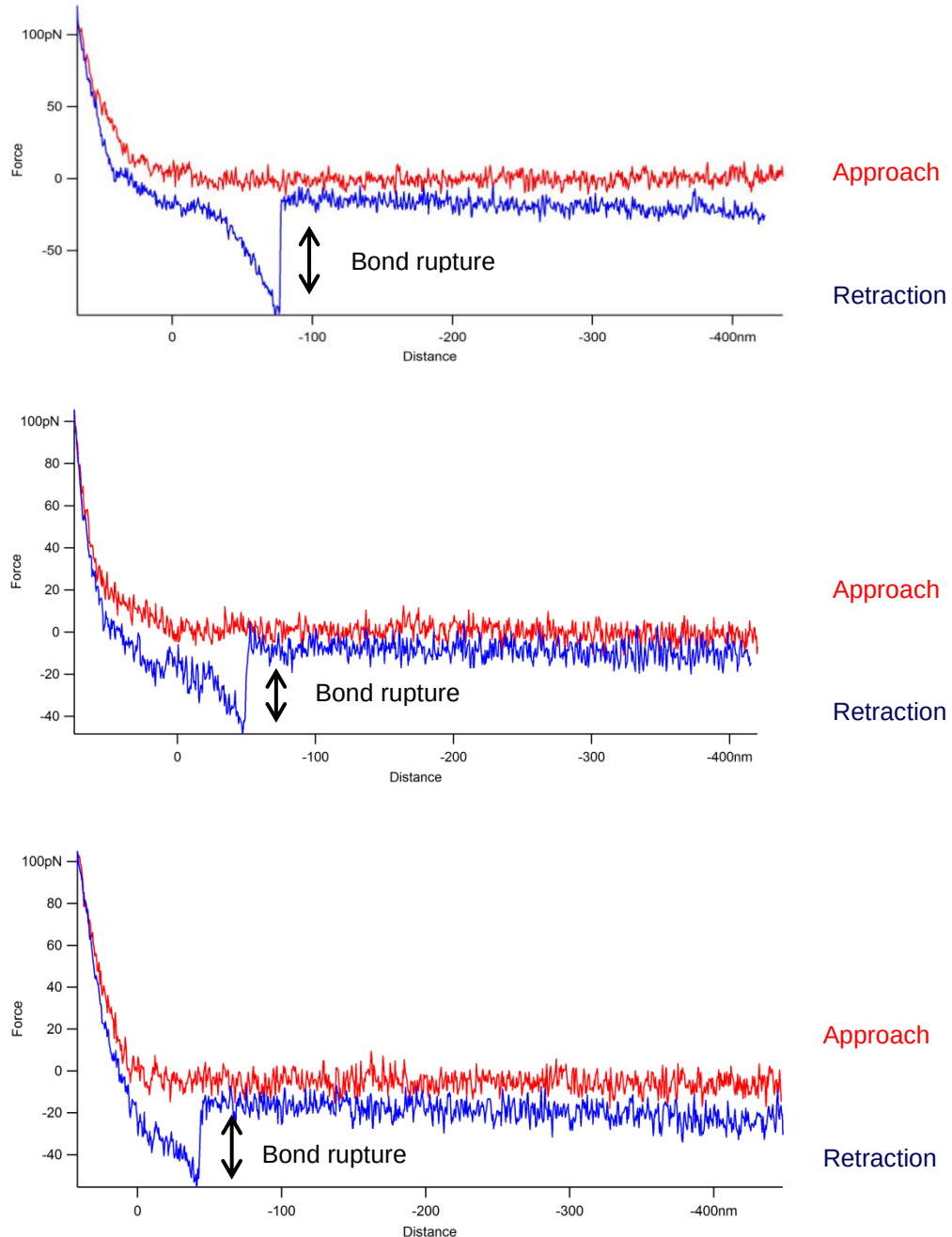


**Figure 3.3. Worked example AFM measurement curves of specific interactions between cadherin coated tips and surfaces. The approach of the AFM tip is shown as the red line and the retract motion of the AFM tip is represented by the blue line. As indicated by the arrows in the example curves above, the bond rupture events showed a nonlinear trend preceding single bond rupture and occurred between 0 nm and 150 nm from the silicon surface.**

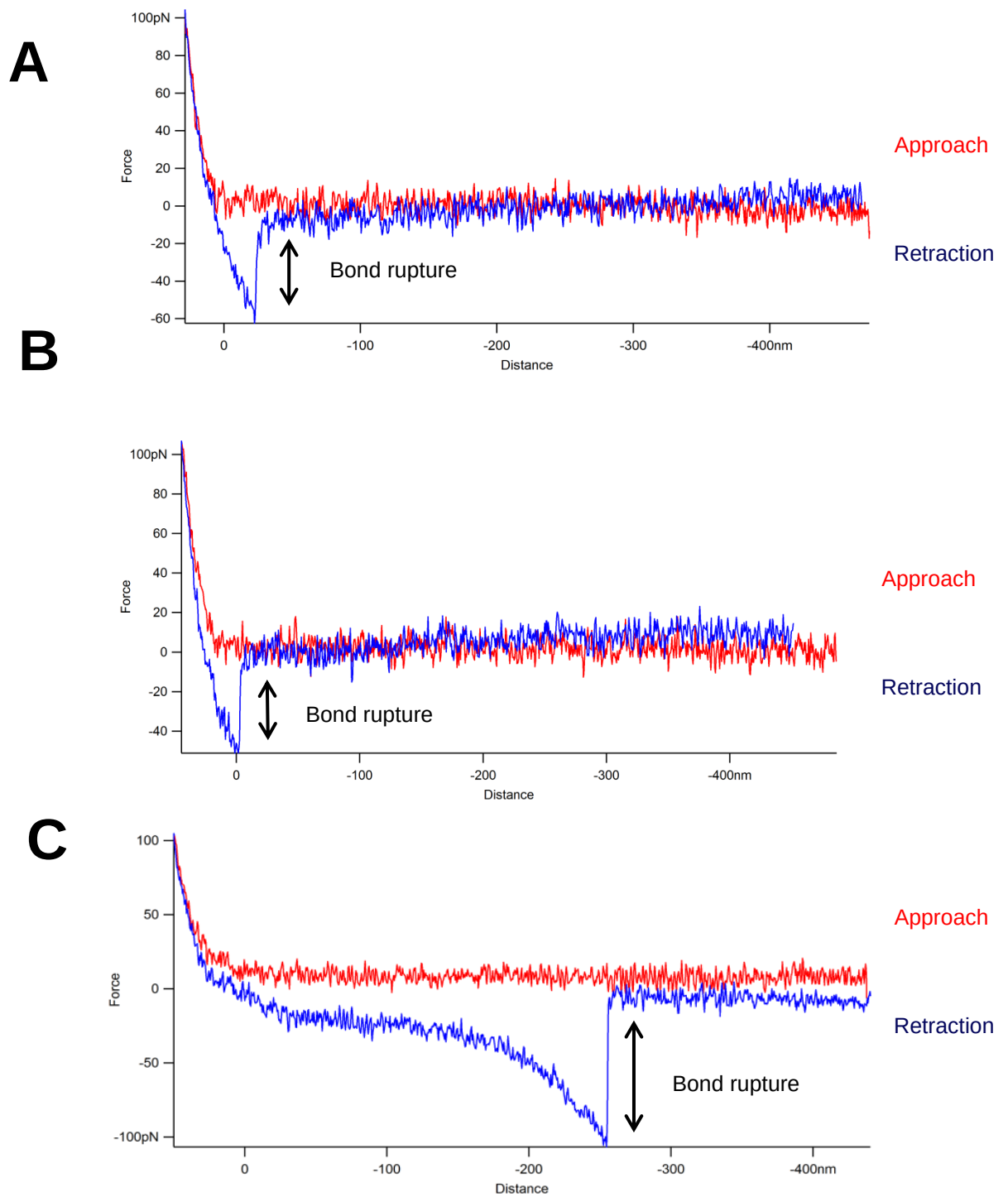


**Figure 3.4. Worked example AFM measurement curves of unspecific interactions between cadherin coated tips and surfaces. The approach of the AFM tip is shown in red and the retract motion by the blue line. (A) Example of unspecific adhesion where the force jump of the force rupture event had a linear appearance prior to bond rupture. (B) Example of unspecific adhesion where the bond rupture event occurred at 0 nm distance from the surface. (C) Example of unspecific adhesion where the adhesion occurred further than 150 nm away from the sample surface.**

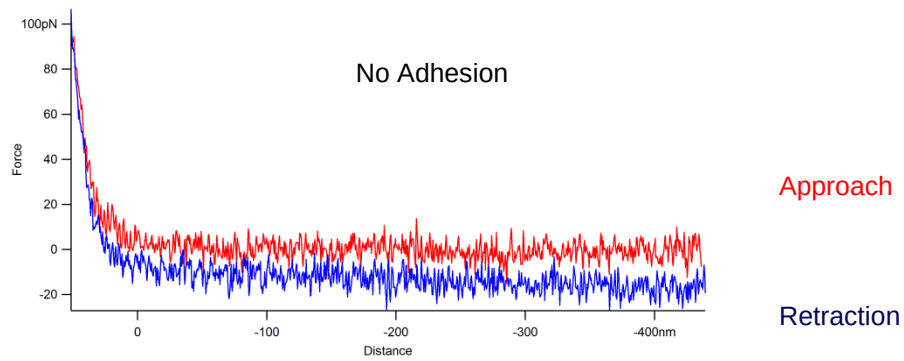
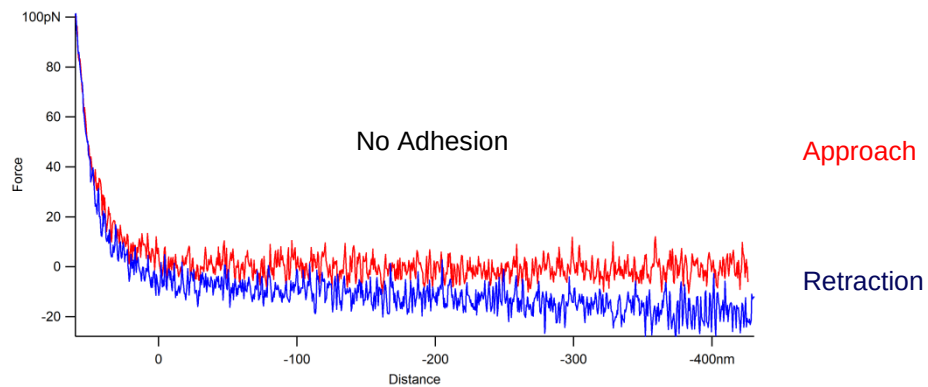
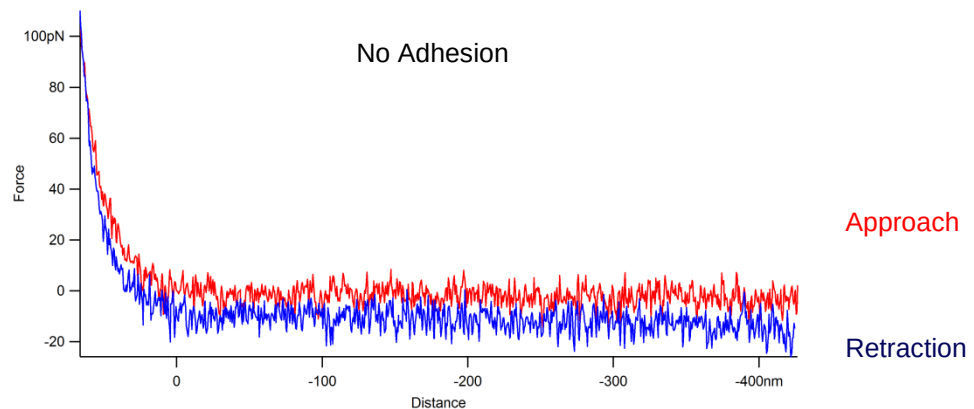
**A****B****C**

**Figure 3.5. Worked example AFM measurement curves showing no interactions between cadherin coated tips and surfaces. The approach of the AFM tip is shown in red and the retract motion by the blue trace. The single bond rupture events that occurred during specific and unspecific interactions are absent and the AFM tip simply approached the sample surface and was then retracted to its default position without any adhesion.**

measuring the adhesion force and calculating their frequency using the Igor Pro software (Wavemetrics). The frequency of specific adhesion events was then determined as the ratio between the number of force curves showing an adhesion event to the total number of force curves recorded. From the distribution of specific rupture forces the most frequently occurring rupture force (the mode force) was determined using the in-house developed software FDist<sup>63, 109</sup> (see Chapter II, Section 2.5.). The data was then plotted as a histogram with a bin size of 10 pN to show the distribution of the specific rupture force magnitudes centred around the mode force (Figure 3.6.).

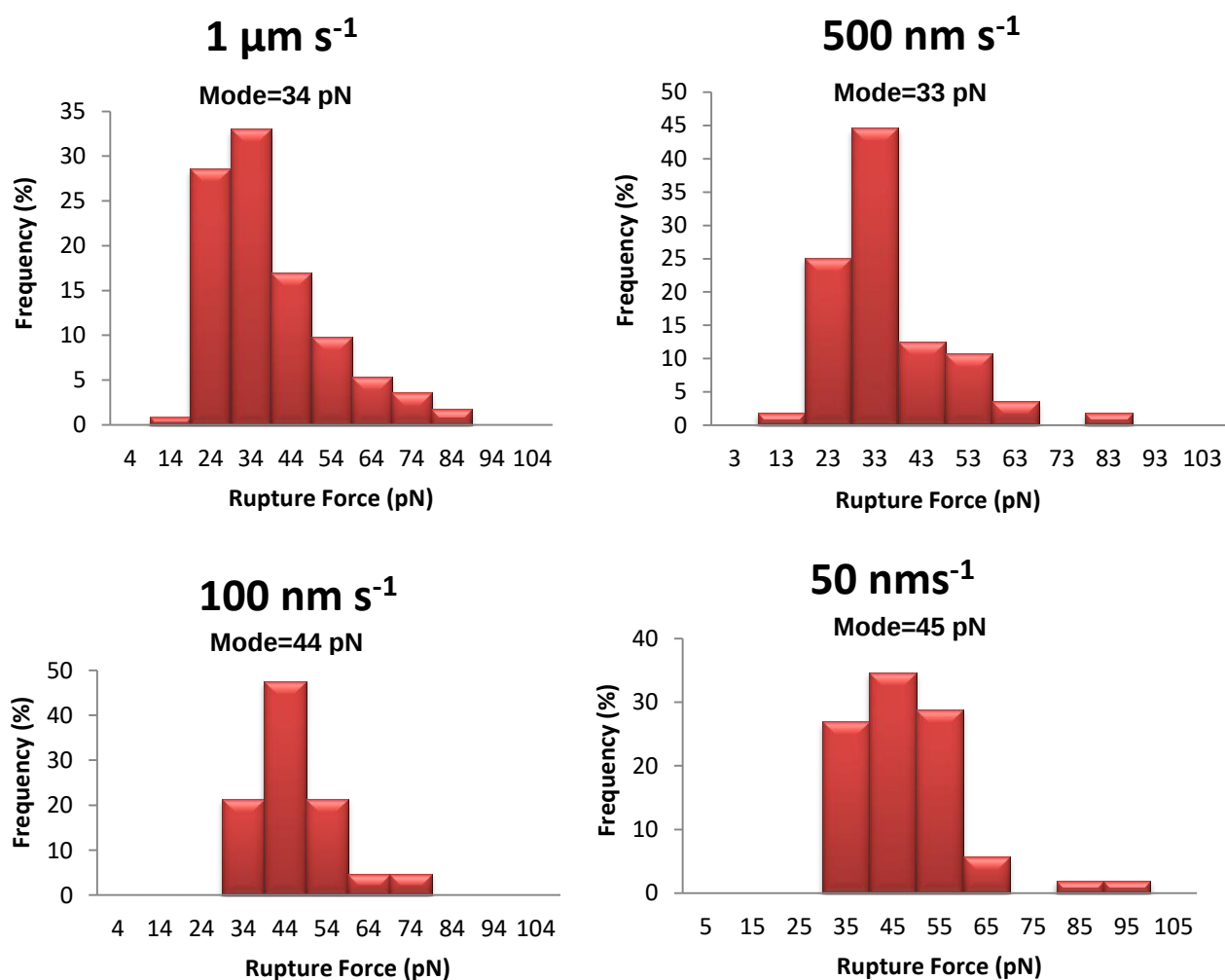


Figure 3.6. Statistical analysis of the measured rupture forces measured between E-cadherin functionalized AFM tips and silicon surfaces at tip speeds of 1  $\mu\text{m s}^{-1}$ , 500  $\text{nm s}^{-1}$ , 100  $\text{nm s}^{-1}$  and 50  $\text{nm s}^{-1}$ . The specific forces were fitted using the in-house developed program Fdist<sup>63, 109</sup> to determine the mode force of the distribution (see Chapter II, Section 2.5.). The data was then plotted as a histogram with a bin size of 10 pN to show the distribution of the specific rupture force magnitudes centred around the mode force.

With decreasing tip speeds the mode force values of specific bond rupture forces increased. At  $1 \mu\text{m s}^{-1}$  the modal rupture force was determined to be 34 pN whereas at  $50 \mu\text{m s}^{-1}$  the modal rupture force increased to 45 pN. At the end of the experiment, in order to demonstrate the specificity of the interaction under investigation the cadherin specific blocking agent EGTA was added in order to block cadherin interactions. The measurement was then repeated again 100 times with and without EGTA, at three different positions on the silicon surface. Addition of EGTA resulted in a significant decline in specific cadherin bond formation. Figure 3.7. shows how the frequency of specific cadherin adhesion events dropped in response to the EGTA treatment from 8.4% to 1.3%.

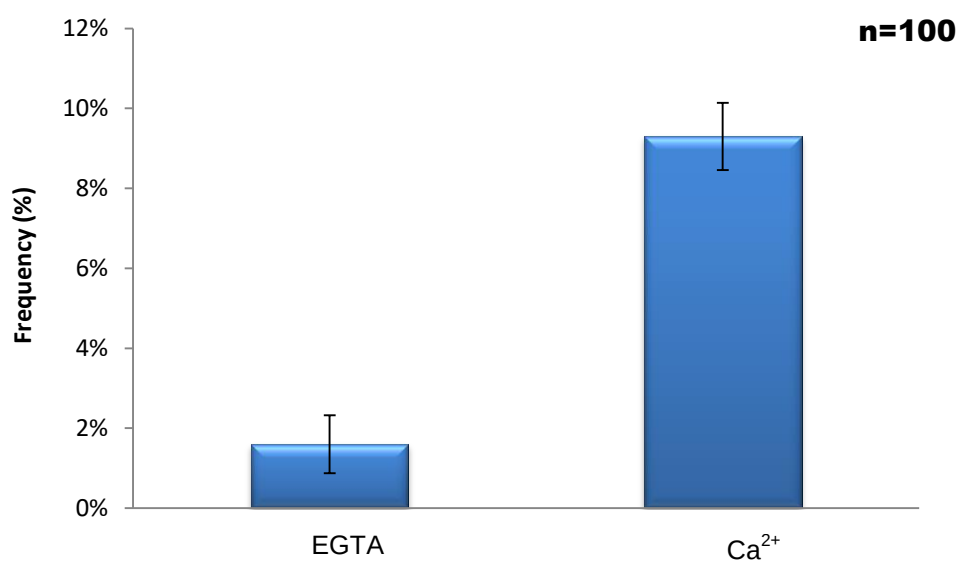


Figure 3.7. Control for the cadherin bond rupture experiment. Data shown represent the frequency of specific rupture force values measured in 100 force measurements using the same AFM tip. In the presence of EGTA, the frequency of specific binding events was greatly reduced presumably due to a change in the structure of the cadherin ectodomains in the induced absence of  $\text{Ca}^{2+}$  ions which allowed them to assume configurations that are unfavourable to bond formations (See Section 3.3.2 below). Error bars shown represent the confidence interval based on the margin of error.

Finally the spring constant of the AFM tip was determined at the end of the experiment as described in Chapter II, Section 2.3.

### **3.3.2. Specificity test of E-cadherin interactions**

In order to clearly show that the observed specific adhesion events were indeed due to bond rupture events between cadherin molecules, force measurement experiments were carried out in the presence of specific cadherin binding inhibitors using the same AFM tip. The  $\text{Ca}^{2+}$  chelating agents EDTA and EGTA were chosen as inhibitors of cadherin interactions as they are known to bind and remove free calcium ions from solutions and cause cadherin ectodomains to change from their rigid rod like structure to their more flexible, amorphous configuration, thereby making it easier for them to assume orientations in which they are unable to interact with opposing cadherin molecules (see Fig.3.1.C) <sup>128</sup>.

For the particular experiment shown, force curves were recorded using the AFM, at  $1 \mu\text{m s}^{-1}$  over a distance of 500 nm using the same AFM tip functionalized with E-cadherin for all experiments (Fig.3.8.A-C). The tip-surface interaction was measured 500 times, at different positions on the silicon wafer for each cadherin blocking agent. In the presence of EGTA and EDTA the frequency of cadherin adhesion events was always substantially reduced (Fig.3.9.A). The chelating agent EGTA was identified here as a more potent inhibitor of cadherin interactions compared to EDTA, which can be attributed to its higher affinity for  $\text{Ca}^{2+}$  ions <sup>129</sup>. The specific binding frequency decreased from 8.4% (no treatment) to 4.5% (EDTA) and 1.6% (EGTA).

Furthermore, an E- cadherin specific antibody was added to the solution in order to prevent specific cadherin bond formation. The addition of this antibody resulted in a significant decline in specific cadherin bond formation. The frequency of specific cadherin adhesion events dropped in response to the antibody treatment from 8.4% to 1.3%. The addition of an antibody specific for the N-cadherin (the cadherin type not functionalized to AFM tip or sample surface) had no inhibitory effect (10.35%).. The cadherin bond inhibitors mentioned above did, however, not in general affect the magnitude of specific rupture forces, except for measurements carried out in EGTA which showed an increase in rupture force compared to measurements in the presence of other binding inhibitors (Fig.3.9.B) which could also be reflective of the limited amount of force values available statistical evaluation.

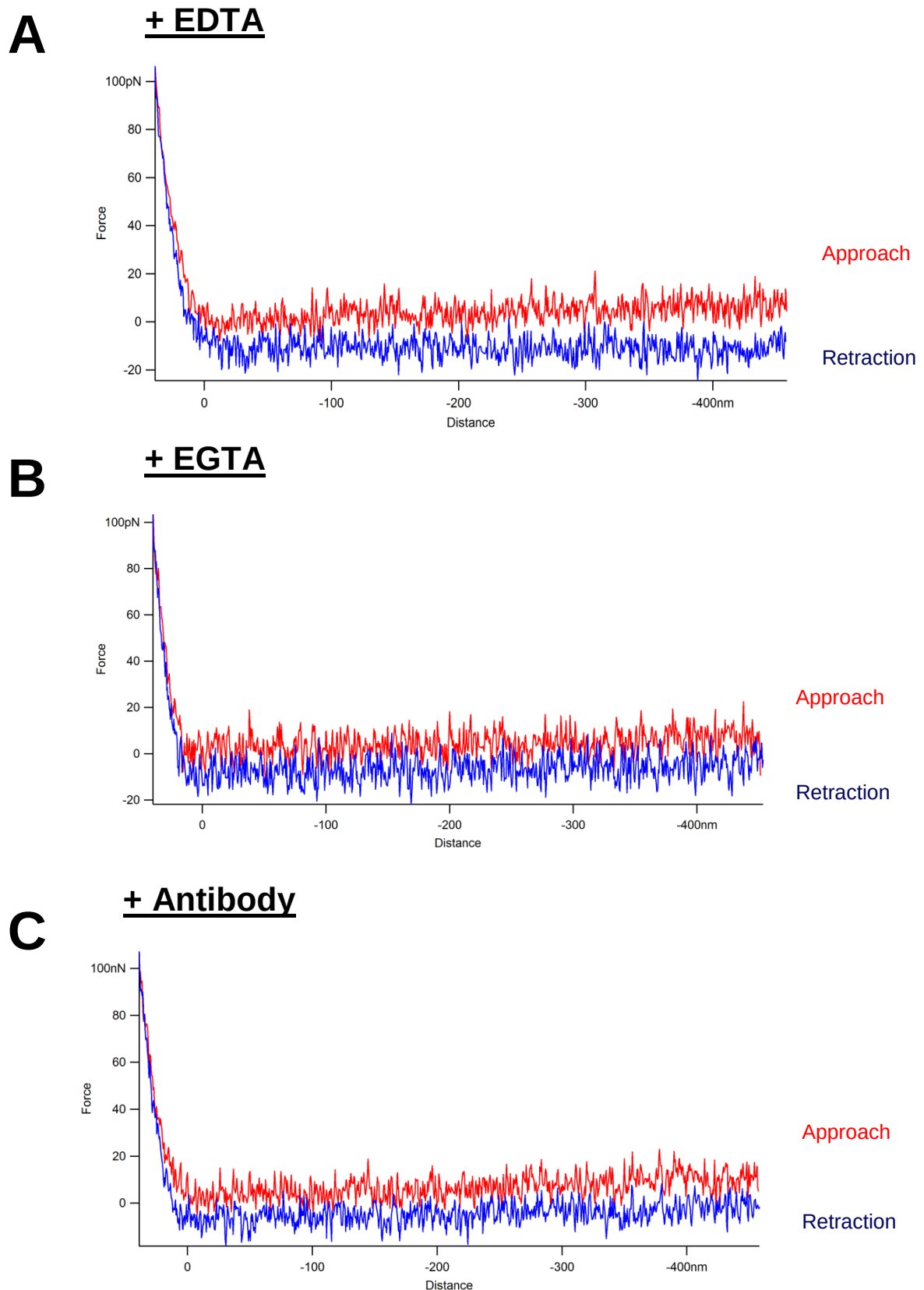


**Figure 3.8. Specificity test for cadherin bond rupture experiments. (A) EDTA force curve obtained from a rupture force measurement in the presence of EDTA. No bond formations could be observed in these measurements. (B) Force curve obtained from a rupture force measurement in the presence of EGTA. The induced absence of  $\text{Ca}^{2+}$  ions greatly inhibited cadherin bond formation. (C) Force curve obtained from a rupture force measurement in the presence of an E-cadherin specific antibody. E-cadherin specific binding was substantially reduced in the presence of an antibody specific for E-cadherin.**

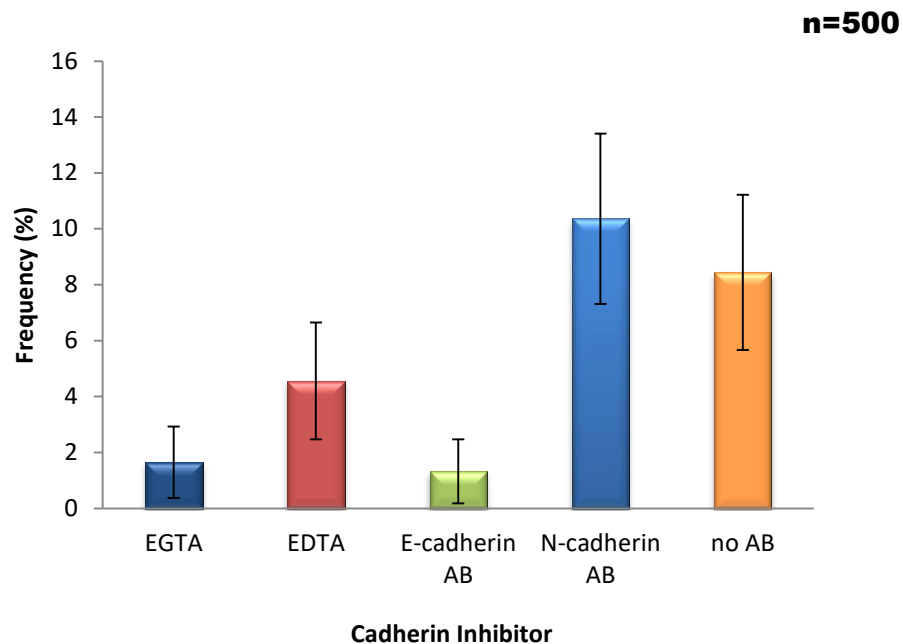
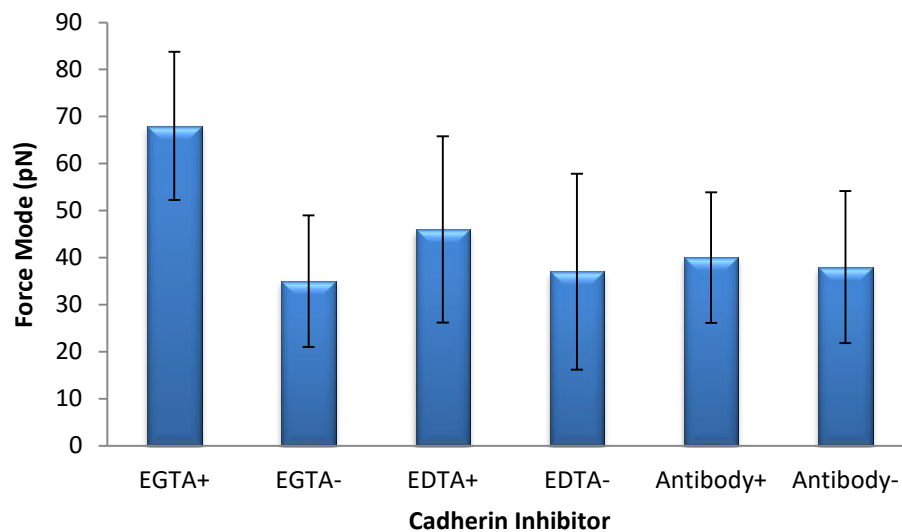
**A****B**

**Figure 3.9. Impact of cadherin binding inhibitors on E-cadherin binding.** Force curves were recorded using the AFM, at  $1 \mu\text{m s}^{-1}$  over a distance of 500 nm using the same AFM tip functionalized with E-cadherin for all experiments. The tip-surface interaction was measured 500 times, at different positions for each treatment on the silicon wafer. Data shown represent the specific rupture values measured in the experiments. (A) Absence of  $\text{Ca}^{2+}$  ions induced by  $\text{Ca}^{2+}$  ion chelators EGTA and EDTA, effectively inhibited cadherin binding. In the presence of an antibody specific for E-cadherin, bond formation was greatly reduced. An antibody specific for N-cadherin showed no inhibitory effect on the frequency of cadherin binding. Error bars shown represent the confidence interval based on the margin of error. (B) Magnitude of rupture forces of E-cadherin bonds did not change significantly in response to cadherin binding inhibitors. The increase in bond rupture force for EGTA could also be reflective of the limited amount of force values available for statistical evaluation. Error bars show the standard deviation of measured rupture forces.

### 3.3.3. Effect of tip speed on E-cadherin interactions

In order to obtain dynamic force spectra to investigate cadherin interactions, cadherin bond rupture forces were measured at four different tip speeds ( $1\ \mu\text{m s}^{-1}$ ,  $500\ \text{nm s}^{-1}$ ,  $100\ \text{nm s}^{-1}$ ,  $50\ \text{nm s}^{-1}$ ). The tip-surface interactions were measured 500 times, for three different AFM tips, at three different positions on the functionalized silicon surface. As shown in Figure 3.10.A. the force required to rupture cadherin bonds decreased when the tip speed was increased. The rupture forces had their highest values at  $50\ \text{nm s}^{-1}$  where forces of up to 48 pN were required to rupture formed cadherin bonds. As the tip speed was increased up to a maximum of  $1\ \mu\text{m s}^{-1}$  the rupture forces of cadherin bonds decreased to values as low as 26 pN.

Our results thus differ from those of other DFS studies in that the force required to rupture cadherin bonds decreased with increasing tip speeds<sup>131</sup>. Because modal rupture forces decreased rather than increased with increasing tip speeds it was not possible to use the data obtained to make assumptions about the energy landscape as described by standard developed DFS theories based on models such as those by e.g. Evans and Ritchie<sup>32, 34, 64</sup> (see Chapter II, Section 2.5.). The observed trend can be attributed to the fact that the tip dwell time on the surface was not set as a constant value (see Section 3.3.4.). The contact time, therefore, varied according to the tip speed being used. e.g. at slower tip speeds the AFM tip and the surface stayed in contact for longer periods of time. A possible explanation for the increase in observed rupture force at slower tip speeds with longer contact times could be that in our case increasing numbers of cadherin subdomains form contacts with one another, leading to the formation of increasingly stronger bonds

between cadherin molecules. Initially only the EC1 and EC2 subdomain at the N-terminal end of opposing cadherin molecules interact and form quick, low strength bonds seen at fast tip speeds e.g.  $1 \mu\text{m s}^{-1}$  with short contact times, subsequently, stronger bonds form that eventually involve all five (EC1-EC5) cadherin subunits and a full overlap of the full ectodomains of two cadherins engaged in a *trans* interaction at slower tip speeds e.g.  $50 \text{ nm s}^{-1}$ .

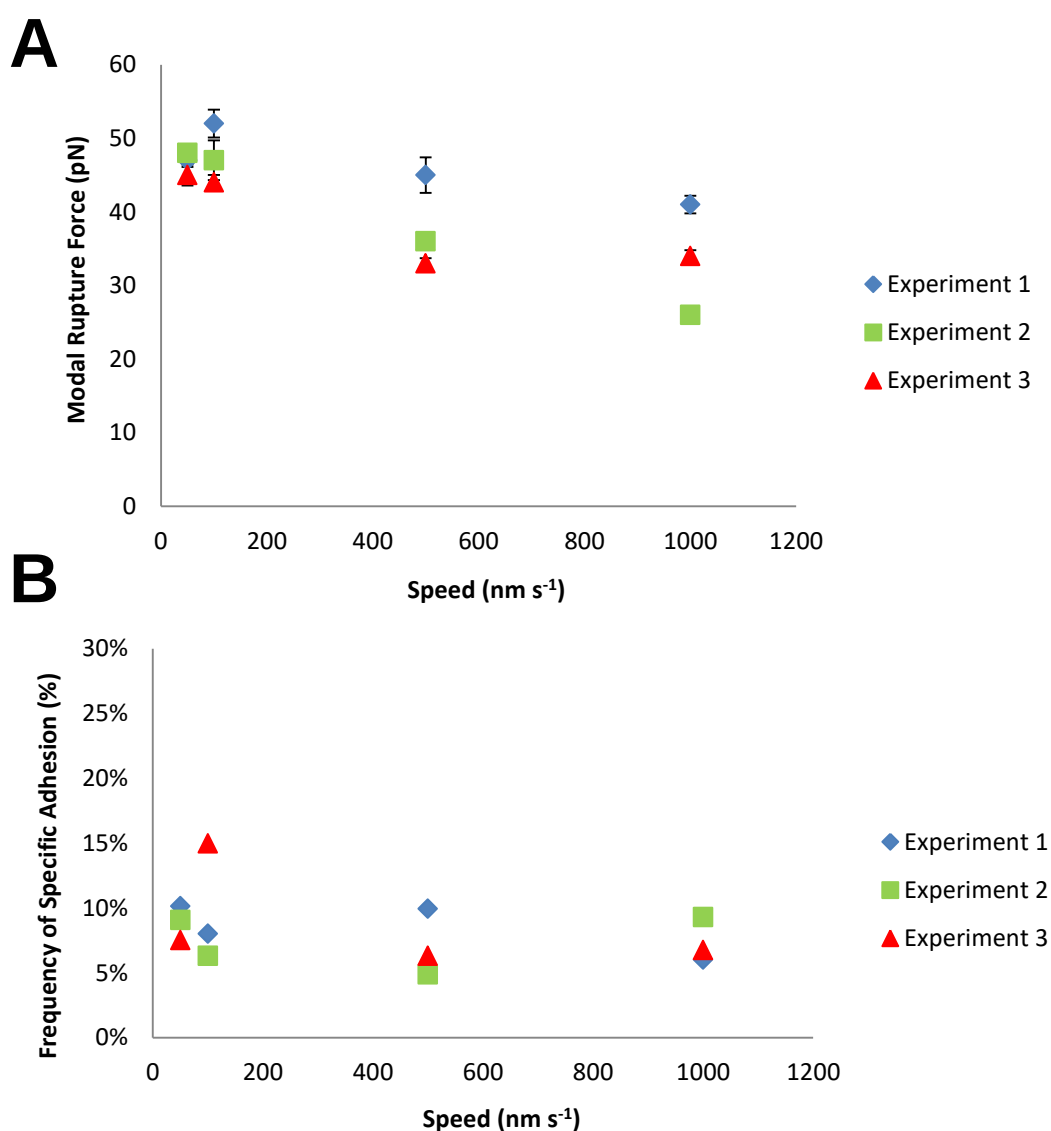


**Figure 3.10. Rupture force dependence on the approach/retract speed of the AFM tip.** Force measurements were carried out for three AFM tips, at four different tip speeds ( $1 \mu\text{m s}^{-1}$ ,  $500 \text{ nm s}^{-1}$ ,  $100 \text{ nm s}^{-1}$ ,  $50 \text{ nm s}^{-1}$ ). Tip-surface interactions were measured and recorded 500 times at three different positions over a distance of 500 nm from the surface for each AFM tip. Data shown represent the specific rupture force values measured in the experiments. (A) As the tip speed was increased rupture forces between E-cadherin molecules decreased. Error bars shown represent the standard deviation of the modal value of recorded forces. (B) The frequency at which specific binding between E-cadherin molecules occurred showed no significant change in response to changing tip speeds.

This multistate binding mechanism of cadherins would be consistent with observations by Leckband *et al.* in force spectroscopy studies of cadherin interactions using the BFP and several other cadherin adhesion studies mentioned above <sup>10, 12, 15, 117</sup>. Another possible explanation could be that more individual cadherin molecules were involved to participate in the adhesion event and thus increased the bond strength of a cadherin contact.

Although the force required to rupture cadherin bonds was found to decrease with increasing tip speeds, the frequency with which specific adhesion events occurred, varied only slightly at any given speed and stayed within a range of 5% to 15% (Fig.3.10.B). The low probability of specific cadherin bond rupture events regardless of changing tip speeds suggested that the rupture events observed were predominantly due to single bond rupture events. According to Poisson statistics as long as the frequency of specific adhesion events is less than 30 %, >80% of the rupture events observed are due to the rupture of single molecular bonds <sup>85, 86</sup>. The bond rupture events were thus likely caused by different types of single bonds rather than multiple independent bonds unbinding simultaneously. These observed single bonds could have been the result of single cadherin domains binding together or cadherin ectodomains could have cooperated as “patches” to form a single molecular bond.

To further investigate the molecular nature of the cadherin interactions under investigation and how the increase in rupture force was linked to the contact time, a set of experiments was performed where the tip dwell time was kept constant for defined time intervals (e.g. 1 s, 5 s etc.).

### **3.3.4. Effect of contact time on E-cadherin interactions**

To investigate the effect of contact time on cadherin bond formation force curves were recorded using the AFM at three different tip dwell times (1 s, 5 s, 10 s) with 100 force curves recorded for each dwell time, at three different positions on the silicon wafer. Keeping the dwell time constant for defined time intervals lead to an increase in rupture events observed per adhesion curve as the cantilever retracted from the surface (Fig.3.11.). The number of observed rupture “steps” increased the longer the tip was allowed to dwell on the surface. (Fig.3.12.A). The largest number of rupture events was observed, in curves recorded with 10 s contact time. Contrary to this increase in rupture events, the frequency of adhesion events i.e. how often any adhesion between AFM tip and the surface occurred decreased directly with an increase in tip contact time (Fig.3.12.B).

The increase in rupture steps per retraction curve seen at longer tip dwell times could have been caused by the formation of multiple cadherin bonds which were being broken sequentially based on the high frequency of adhesion events (>30%) at any of the selected tip dwell times or unspecific binding events. Multi step retraction curves were therefore too complex to be analysed using DFS because the origin of observed rupture events could not easily be determined.

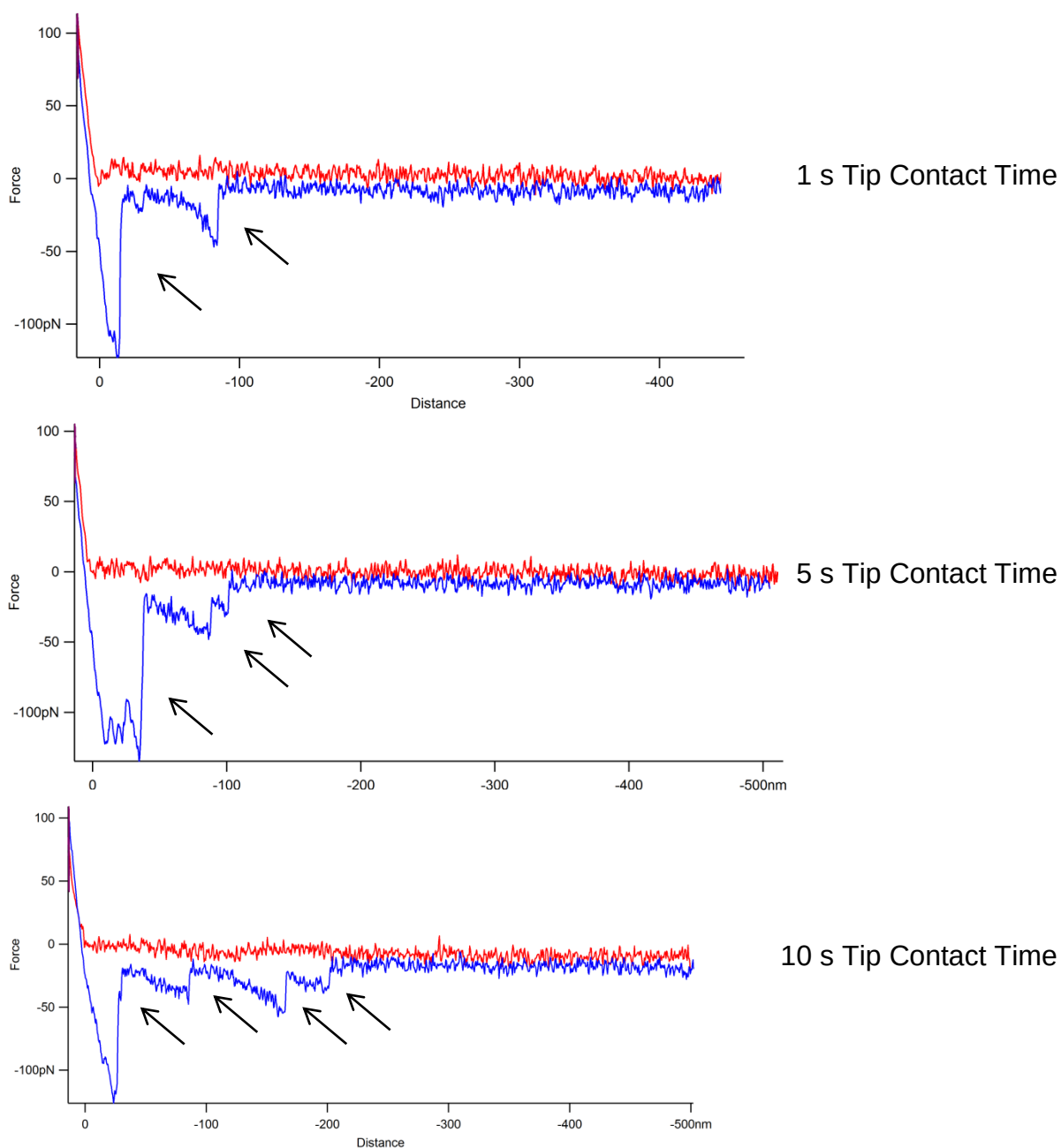


**Figure 3.11. Effects of prolonged tip dwell times on E-cadherin interactions. A longer dwell time of the functionalized AFM tip on the sample surface led to an increase in the number of bond rupture events. The black arrows point towards individual bond rupture events. The force curves had a stairway like appearance with many force steps in one retraction curve.**

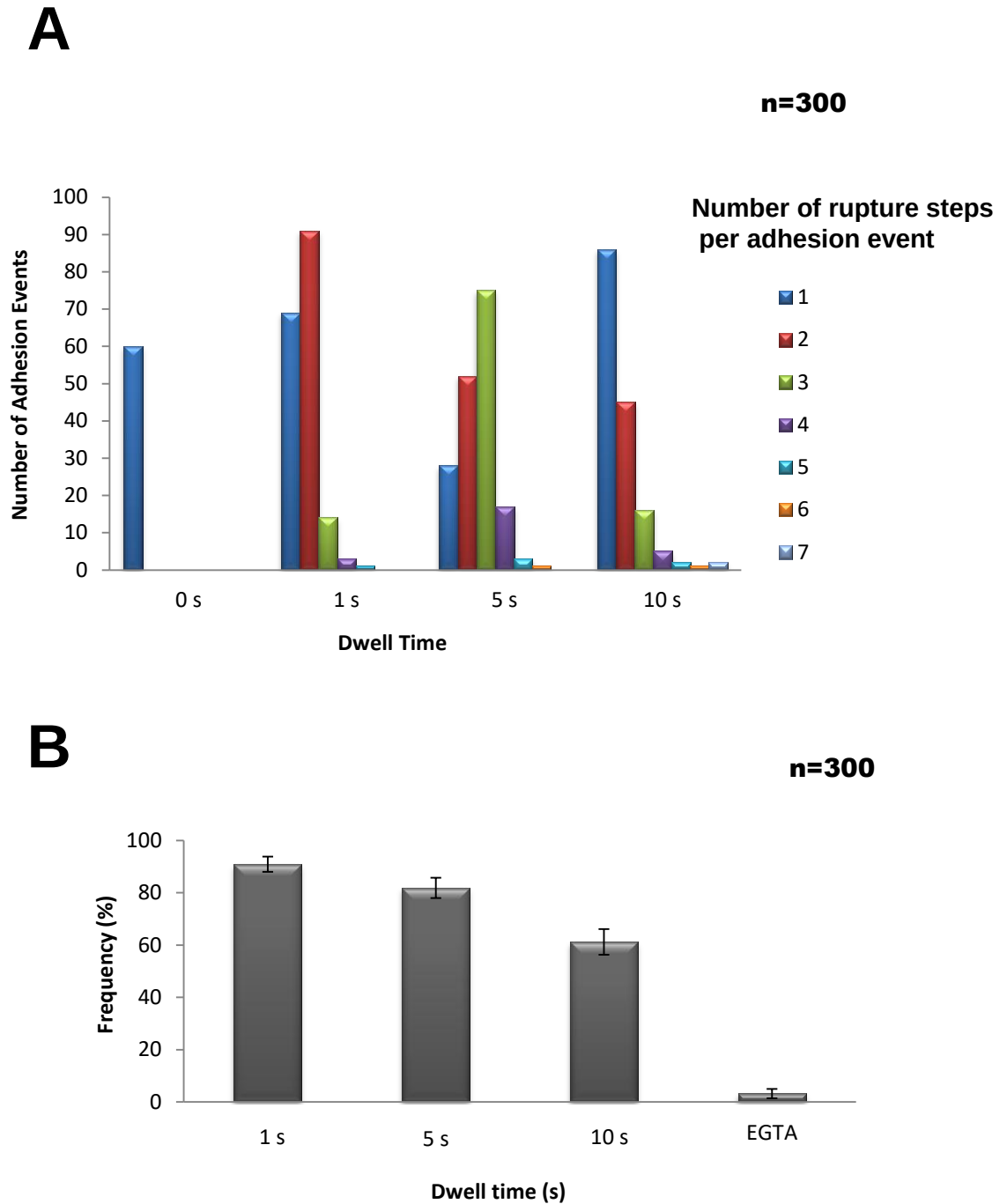


**Figure 3.12. Effects of prolonged tip dwell times on E-cadherin rupture events. (A)** With increasing tip dwell time the number of rupture steps per adhesion curve increased. **(B)** By increasing the tip dwell time the frequency of adhesion events decreased although the observed frequencies were too high to exclude the possibility of multiple cadherin bond formations. Error bars shown represent the confidence interval based on the margin of error.

Since the total contact time of tip and surface depends on tip speed in addition to dwell time the effect of tip speed with 0 dwell time was also investigated. In these experiments the time the tip dwells on the sample surface depended only on the approach and retraction speed of the AFM tip during the measurement. With no pre-set dwell time, all retraction curves had only one rupture step in them (Fig.3.12.A), presumably due to an optimal combination of cadherin density and AFM tip dwell time at the respective tip speed. The single rupture steps suggested that only single cadherin bonds were forming and that these interactions were being ruptured. The single bond nature of cadherin interactions in retraction curves with no dwell at the surface was further supported by the frequency analysis shown in Figure 3.10.B (Section 3.3.3.). Here, the frequency of specific adhesion events was low (5% to 15%) and did not vary according to tip speed, which is another indicator for single bond formations according to Poisson<sup>85, 86</sup> statistics.

The amount of tip-surface contact time, at zero pre-set tip dwell time, was estimated by considering the following assumptions: First, the time the tip is in contact with the surface can be viewed as dependent on the contact force ( $F_c$ ), the spring constant of the AFM cantilever  $k$  and the tip speed ( $V$ ) in the force-distance curve of any given approach and retract cycle of a single AFM force measurement. At the contact force  $F_c$  the cantilever is deflected by  $F_c/k$ . The time taken to deflect  $F_c/k$  distance is  $F_c/k V$ . Since the lever approaches and retracts with the same speed the total contact time is given by  $2 F_c/kV$

If the dwell time of the AFM tip on the sample surface is set to 0 seconds it follows that the total duration of the contact time is given by:

$$t_{contact} = \frac{F_C}{k_{V_A}} + t_{dwell} + \frac{F_C}{k_{V_R}}$$

$$V_{Approach} = V_{Retract}$$

$$t_{contact} = \frac{2Fc}{kV} + t_{dwell}$$

Equation 3.1.

The total amount of contact time of the tip with the surface decreased as the tip speed was increased, as shown in Figure 3.13.A. At the slowest of the tested tip speeds ( $50 \text{ nm s}^{-1}$ ) the tip stayed in contact with the surface for a maximum contact time of 0.44 seconds, whereas at the fastest of the tested tip speeds ( $1 \text{ } \mu\text{m s}^{-1}$ ) the tip stayed in contact with the surface for a minimum of 0.014 seconds. The force required to rupture E-cadherin bonds was found to increase with increasing contact time (Fig.3.13.B).

In summary these findings indicated that in order to probe single molecular bonds of cadherin molecules with DFS it was necessary to keep the dwell time at zero. An increase in contact time at slower tip speeds lead to an increase in rupture force possibly due to optimal arrangements of interacting cadherin subunits of single cadherin ectodomains. This means that these results differed from other DFS studies in that the rupture force increased with slower tip speeds <sup>131</sup>. In a typical DFS experiment the rupture force usually increases linearly as a function of the logarithm of the loading rate <sup>60</sup> (See Chapter II, Section 2.5.). The logarithmic dependence of the mode rupture

force observed in similar studies could be attributed to the conserved duration of set dwell time of the AFM tip. In those studies the dwell time was always set to be at least 100 milliseconds in addition to the contact time resulting from the chosen tip speed <sup>12, 118, 119</sup>. This could have affected the molecular bond by giving it an additional constant time of contact which was absent in the experiment carried out for the work of this thesis. Furthermore the rigidity of the covalent silane surface functionalization, employed for the work of this thesis, could have contributed to this effect by reducing the binding probability at shorter contact times.

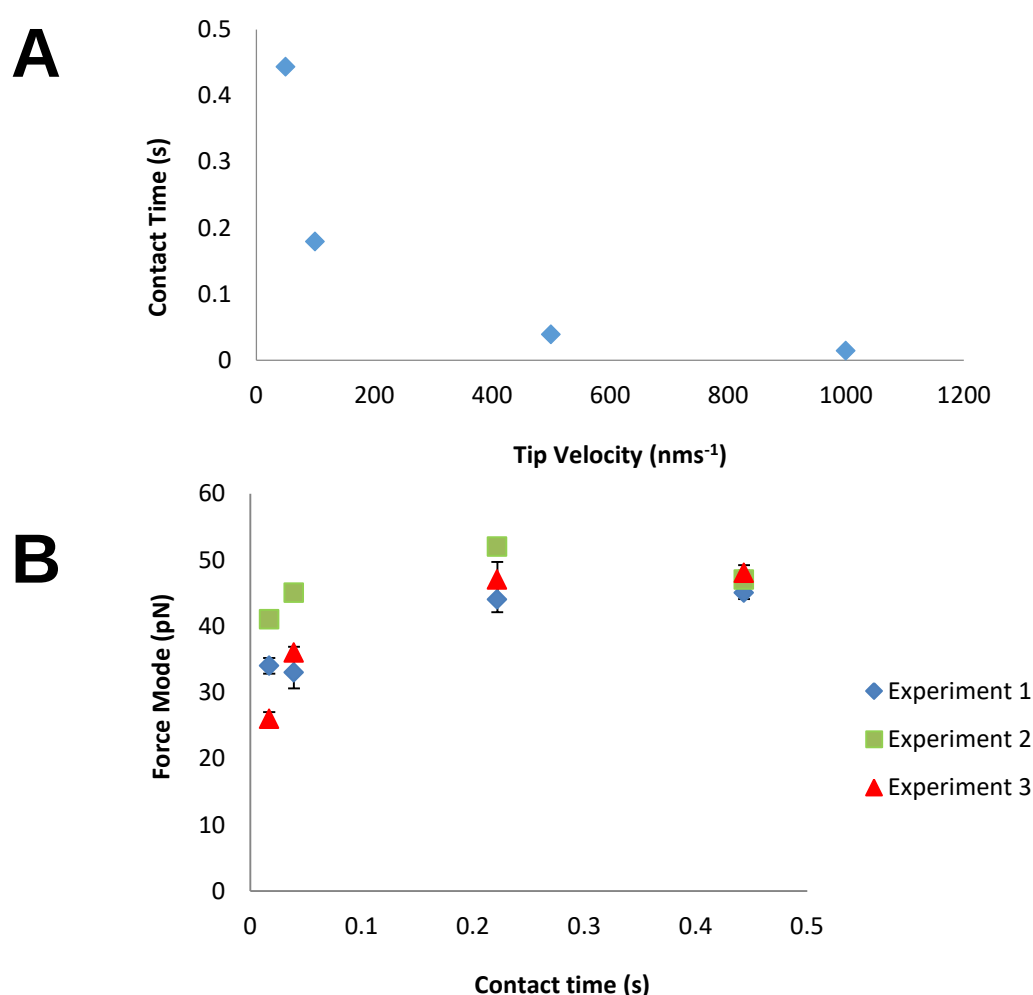


Figure 3.13. Dependence of contact time on tip speed. Force measurements were carried out at four different tip speeds ( $1 \mu\text{m s}^{-1}$ ,  $500 \text{ nm s}^{-1}$ ,  $100 \text{ nm s}^{-1}$ ,  $50 \text{ nm s}^{-1}$ ). Tip-surface interactions were measured and recorded 500 times at three different positions over a distance of 500 nm from the surface for each AFM tip. Data shown represent the specific rupture force values measured in three repetitions of the same experiment using three different AFM tips. (A) Fast tip approach/retraction speeds led to shorter contact times between the AFM tip and the sample surface. (B) E-cadherin bond rupture forces in dependence on tip-surface contact time. As contact times increased the E-cadherin bonds became stronger and required larger forces to be disrupted. Error bars shown represent the standard deviation of the modal value of recorded forces.

Despite these differences, as this measurement technique could yield single bond formations which could be blocked by specific cadherin binding antagonists, it was now possible to extend these measurements to other types of cadherin interactions such as homophillic N-cadherin interactions and heterophillic E-cadherin-N-cadherin interactions.

### **3.3.5. N-cadherin interactions**

N-cadherin coated AFM tips and wafers were brought into and out of contact to investigate interactions between N-cadherin ectodomains. The experimental procedure was kept the same as for E-cadherin interactions described above. First, to demonstrate the specificity of the N-cadherin interactions under investigation, force measurements were carried out in the presence of known N-cadherin binding inhibitors such as the  $\text{Ca}^{2+}$  chelating agent EGTA and an antibody specific for N-cadherin (Fig.3.14.A). Force curves were recorded using the AFM at  $1 \mu\text{m s}^{-1}$  over a distance of 500 nm using the same AFM tip functionalized with N-cadherin for all experiments. The tip-surface interaction was measured 500 times at different positions on the silicon wafer for each cadherin blocking agent. Addition of the N-cadherin specific antibody or EGTA reduced the frequency of specific binding events from 7.3% to 0.8% and 1.0%, respectively. Surprisingly, the addition of an antibody specific for E-cadherin also reduced the frequency of specific binding events, although, not to the same extend as the N-cadherin specific antibody from 7.3% to 3.9%. This binding behaviour could be caused by unspecific recognitions of N-cadherin by the E-cadherin specific antibody due to the high sequence similarity of E-and N-cadherin ectodomains <sup>130</sup>. The modal rupture

force of N-cadherin interactions did not change significantly in response to the E-cadherin specific antibody or in response to EGTA (Fig.3.14.B), however the addition of an antibody specific for N-cadherin increased the modal rupture force which could be due to the limited number of force curves available for statistical evaluation.

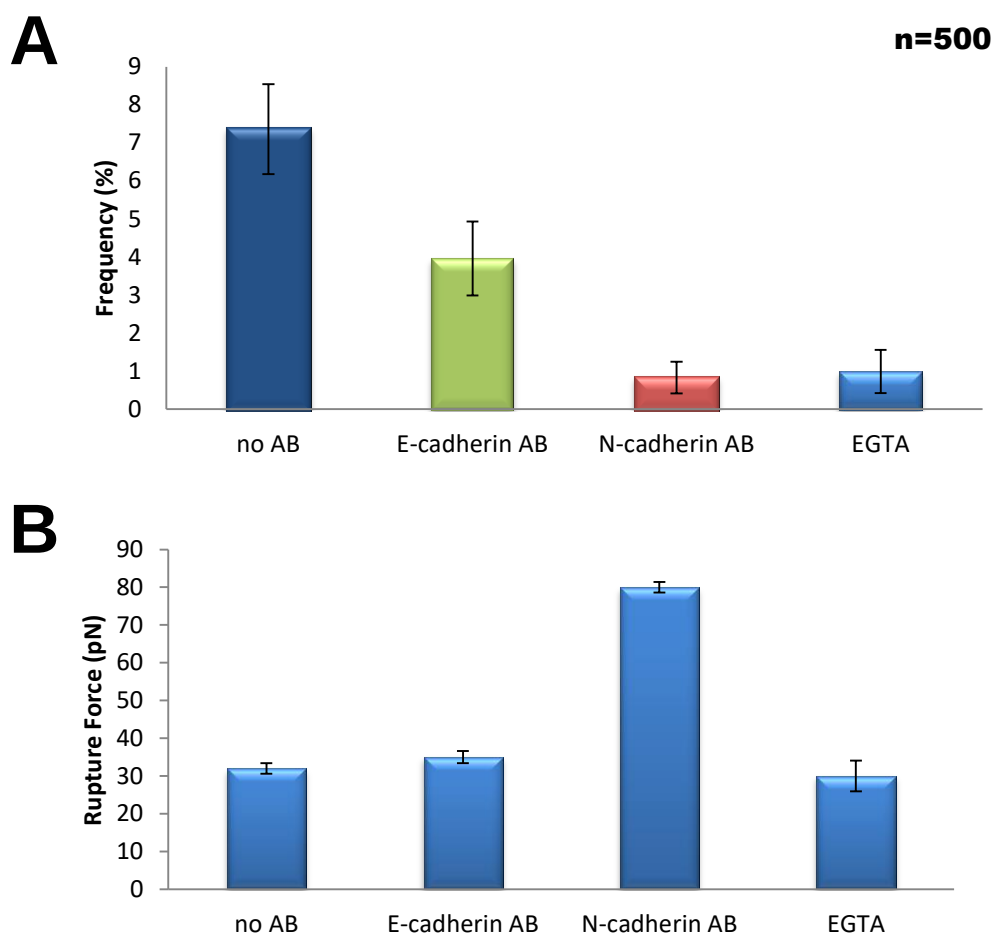


**Figure 3.14. Impact of cadherin binding inhibitors on N-cadherin binding.** Force curves were recorded using the AFM, at  $1 \mu\text{m s}^{-1}$  over a distance of 500 nm using the same AFM tip functionalized with N-cadherin for all experiments. The tip-surface interaction was measured 500 times, at different positions for each treatment on the silicon wafer. Data shown represent the specific rupture force values measured in the experiments. (A) An antibody specific for E-cadherin reduced the frequency of specific adhesion events although not to the same extent as the N-cadherin specific antibody. The reason for this could be unspecific recognitions of N-cadherin by the E-cadherin specific antibody due to the high sequence similarity of E-and N-cadherin ectodomains<sup>130</sup>. Error bars shown represent the confidence interval based on the margin of error. (B) Magnitude of rupture forces of N-cadherin bonds did not change significantly in response to cadherin binding inhibitors. The increase in rupture force seen for the N-cadherin specific antibody is likely due to the small number of rupture curves available for statistical analysis. Error bars show the standard deviation of measured rupture forces.

Figure 3.15.A shows the appearance of an AFM measurement of N-cadherin interactions blocked with an N-cadherin specific antibody. Figure 3.15.B and Figure 3.15.C show the typical appearance of an N-cadherin unbinding curve and rupture force distribution respectively. It can be seen from these images that N-cadherin interactions strongly resembled those of E-cadherin interactions described above (see Section 3.3.1.)

In order to generate force spectra for the interaction between the ectodomains of N-cadherin, force measurements were carried out at four different tip speeds following the same procedure as described for E-cadherin above (Section 3.3.3.). Due to approaching project deadlines this experiment could only be carried out for one AFM tip. As a control, at the end of each experiment, an N-cadherin specific antibody or EGTA was added to the HEPES buffer. Figure 3.16.A shows the measured rupture forces in dependence on the approach/retract speed of the AFM tip. Rupture forces were found to decrease with increasing tip speeds. At the slowest of the tested tip speeds ( $50 \text{ nm s}^{-1}$ ) the mode of the rupture force of N-cadherin bonds reached its highest value ( $60 \text{ pN} \pm 2.6 \text{ pN}$ ). This value was slightly higher compared to E-cadherin bonds at the same speed ( $44 \text{ pN} \pm 0.9 \text{ pN}$ ). The standard error of the mode was estimated using the procedure described in Chapter II, Section 2.5. As the tip approach and retraction speeds were increased from  $50 \text{ nm s}^{-1}$  to  $1 \text{ } \mu\text{m s}^{-1}$  the modes of N-cadherin rupture forces decreased consistently, reaching its lowest value at  $1 \text{ } \mu\text{m s}^{-1}$  of  $32 \text{ pN} \pm 1.4 \text{ pN}$ .

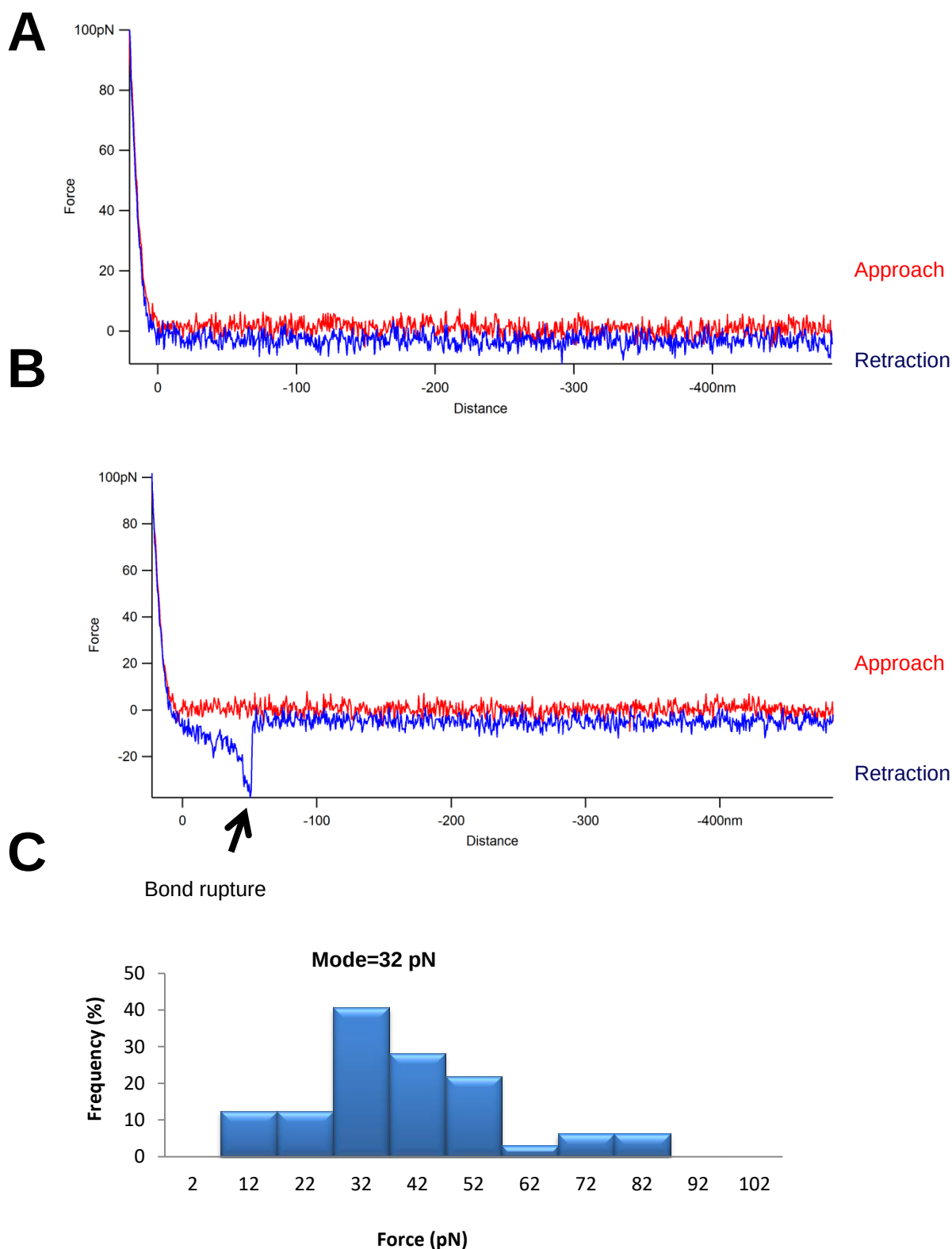


Figure 3.15. AFM measurement of N-cadherin interactions. (A) Blocking experiment of N-cadherin interactions. In the presence of an N-cadherin specific antibody no adhesion between tip and surface occurred. (B) Typical appearance of an N-cadherin force curve for specific adhesion. The extension curve showed nonlinear stretching behaviour preceding bond rupture. (C) Histogram of 1500 bond rupture force values. N-cadherin bond rupture forces shown were measured at a tip velocity of  $1 \mu\text{m s}^{-1}$ . The diagram displayed a distinct maximum at 32 pN. The specific forces were fitted using the in-house developed program Fdist<sup>63, 109</sup> to determine the mode force of the distribution (see Chapter II, Section 2.5.). The data was then plotted as a histogram with a bin size of 10 pN to show the distribution of the specific rupture force magnitudes centred around the mode force.

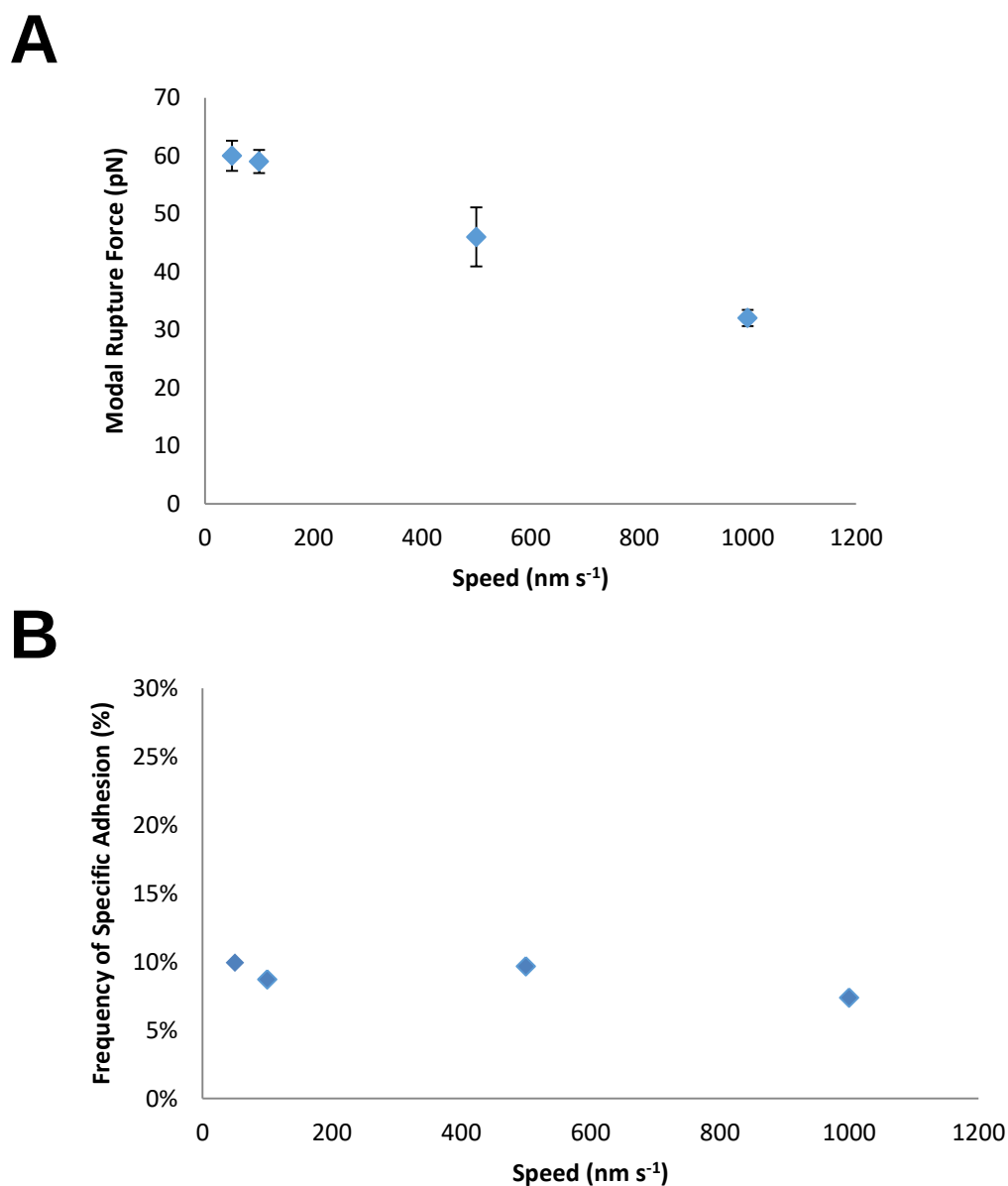


**Figure 3.16. (A) Rupture forces of N-cadherin interactions in dependence on the AFM tip speed. Force measurements were carried out at four different tip speeds ( $1 \mu\text{m s}^{-1}$ ,  $500 \text{ nm s}^{-1}$ ,  $100 \text{ nm s}^{-1}$ ,  $50 \text{ nm s}^{-1}$ ). Tip-surface interactions were measured and recorded 500 times at three different positions over a distance of 500 nm from the surface for one AFM tip. Data shown represent the specific bond rupture force values measured in the experiment. As the tip speed was increased rupture forces of N-cadherin bonds decreased. Error bars shown represent the standard deviation of the modal value of recorded forces. (B) The frequency at which specific binding between N-cadherin molecules occurred showed no significant change in response to changing tip speeds.**

Because the dwell time was kept at zero as in the E-cadherin experiments (see section 3.3.3.) the modal values of the rupture force increased with an increase in contact time in a similar fashion as was observed for E-cadherin interactions. The binding frequency of specific adhesion events remained almost entirely unaffected by the change in tip speed and stayed between 7% and 10%.

Because classical type I cadherins E- and N-cadherin have a high sequence similarity of their EC1-EC5 and EC1 subunits ( 48% / 58% respectively) <sup>130</sup>, it is not surprising that in these experiments E- and N-cadherin appear to behave in a similar manner (e.g. similar rupture forces and speed dependencies).

### **3.3.6. Heterophillic cadherin interactions**

In order to assess the ability of E-and N cadherin ectodomains to undergo heterophillic interactions, force measurements between both types of cadherin were carried out. The experimental procedure was kept the same as for the E- and N-cadherin interactions at different tip speeds as described above (Section 3.3.1/3.3.5. respectively). Due to time constraints this experiment was carried out for one AFM tip only. In these experiments the AFM tip was functionalized with either E- or N cadherin and brought into contact with a sample surface that was functionalized with the same type of cadherin and subsequently with a surface functionalized with the other type of cadherin. First, in order to test the ability of the AFM tips functionalized with E-or N-cadherin to form homophillic bonds they were brought into contact with a surface with the same kind of cadherin and their interaction was then blocked

using EGTA (Fig.3.17.A/B). The frequency of specific binding events in response to this treatment was greatly reduced. This ‘tip test’ demonstrated the functionality of the cadherin ectodomains on the AFM tip. Tips that were validated in this way were then selected for the study of heterophilic cadherin binding. When brought into contact, heterophilic binding between E and N cadherin occurred and displayed binding behaviour that strongly resembled that of homophilic interactions, with force-distance curves that showed nonlinear stretching behaviour preceding bond rupture and a similar distribution of rupture forces (Fig.3.18.A/B).

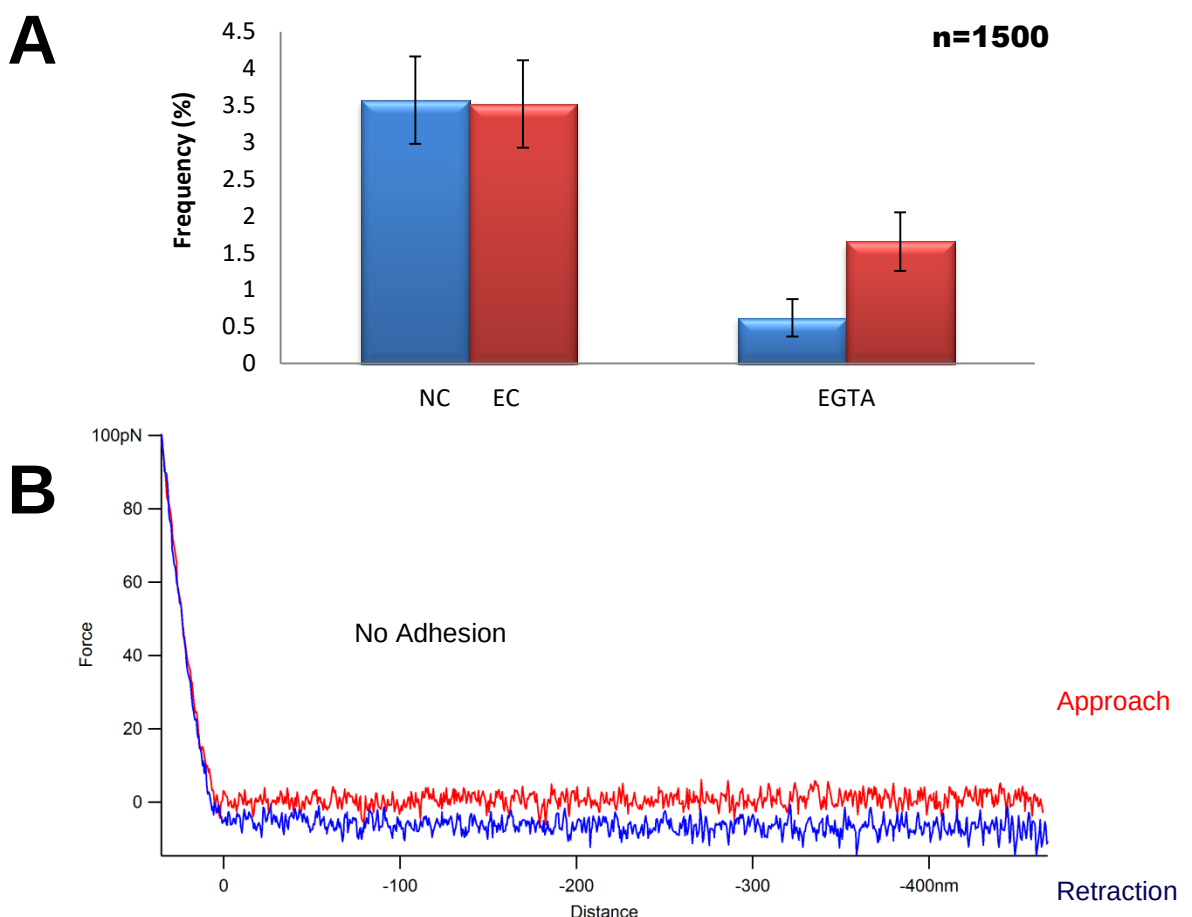


**Figure 3.17. Impact of EGTA on the binding ability of an E- (EC) or N- (NC) cadherin tip to the surface with the same cadherin type. (A)** In the presence of EGTA, the frequency of specific binding events was greatly reduced. This demonstrated the functionality of the E-or N-cadherin ectodomains used for heterophilic binding experiments. Error bars shown represent the confidence interval based on the margin of error. **(B)** Force graph of a blocked E-cadherin interaction. In the presence of the EGTA no adhesion between tip and surface occurred.

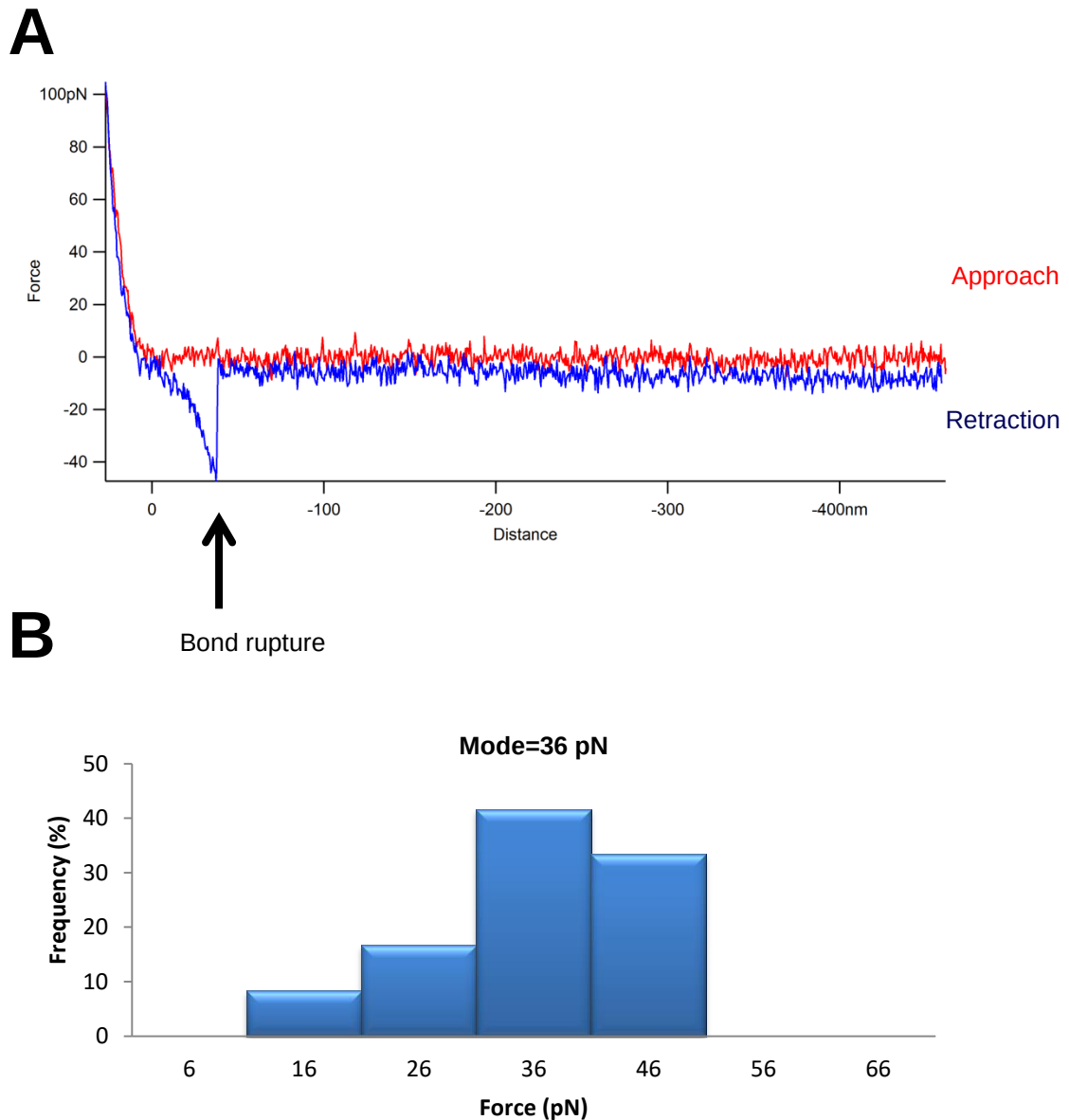


**Figure 3.18. AFM measurement of heterophilic cadherin interactions. (A)** Typical appearance of a heterophilic unbinding curve from a force measurement between an E- cadherin tip and N-cadherin surface. The extension curve showed nonlinear stretching behaviour preceding bond rupture. **(B)** Histogram of 1500 force measurements. Data shown represent the frequency of specific bond rupture force values measured in the experiments. Heterophilic rupture forces shown were measured at a tip velocity of  $1 \mu\text{m s}^{-1}$ . The diagram displayed a distinct maximum at 36 pN. The bond rupture forces were fitted using the in-house developed program Fdist<sup>63, 109</sup> to determine the mode force of the distribution (see Chapter II, Section 2.5.). The data was then plotted as a histogram with a bin size of 10 pN to show the distribution of the specific rupture force magnitudes centred around the mode force.

Rupture force measurement at decreasing tip speeds also showed an overall increase in bond rupture forces at slower velocities (Fig.3.19.A) (Fig.3.20.A).

The rupture forces measured for homophillic and heterophillic interactions had similar magnitudes of rupture forces at different tip speeds. At the slowest of the tested tip speeds ( $50 \text{ nm s}^{-1}$ ) for heterophillic interactions, the E-cadherin tip displayed slightly larger rupture forces compared to the N-cadherin tip ( $72 \text{ pN} \pm 13.3 \text{ pN}$  and  $42 \pm 13.8 \text{ pN}$  respectively). At the fastest of tested tip speeds ( $1 \text{ } \mu\text{m s}^{-1}$ ) the heterophillic rupture forces for the E- and N-cadherin tip were almost identical ( $36 \text{ pN} \pm 1.9 \text{ pN}$  and  $37 \pm 2.9 \text{ pN}$  respectively). The standard error of the mode was estimated using the procedure described in Chapter II, Section 2.5.

Homophillic interactions were probed by changing the functionalized surface to a surface that was functionalized with the same type of cadherin as the AFM tip. The rupture forces of homophillic interactions measured with the E-cadherin tip were overall higher compared to heterophillic interactions apart from rupture forces measured at  $50 \text{ nm s}^{-1}$  where homophillic rupture forces were found to have lower force rupture force values (homophillic  $56 \text{ pN} \pm 2.8 \text{ pN}$ ; heterophillic  $72 \text{ pN} \pm 13.3 \text{ pN}$  ). For the N-cadherin tip, homophillic rupture forces at  $50 \text{ nms}^{-1}$  ( $42 \text{ pN} \pm 4 \text{ pN}$ ) and  $100 \text{ nm s}^{-1}$  ( $36 \text{ pN} \pm 4.4 \text{ pN}$ ) were lower compared to heterophillic interactions ( $50 \text{ pN} \pm 2.8 \text{ pN}$ ,  $43 \text{ pN} \pm 2 \text{ pN}$ ) whereas rupture forces at  $500 \text{ nm s}^{-1}$  and  $1 \text{ } \mu\text{m s}^{-1}$  were higher for homophillic interactions ( $36 \text{ pN} \pm 1.5 \text{ pN}$  and  $46 \text{ pN} \pm 6.9 \text{ pN}$  respectively) compared to heterophillic interactions ( $33 \text{ pN} \pm 1.2 \text{ pN}$  and  $37 \text{ pN} \pm 2.9 \text{ pN}$ ).

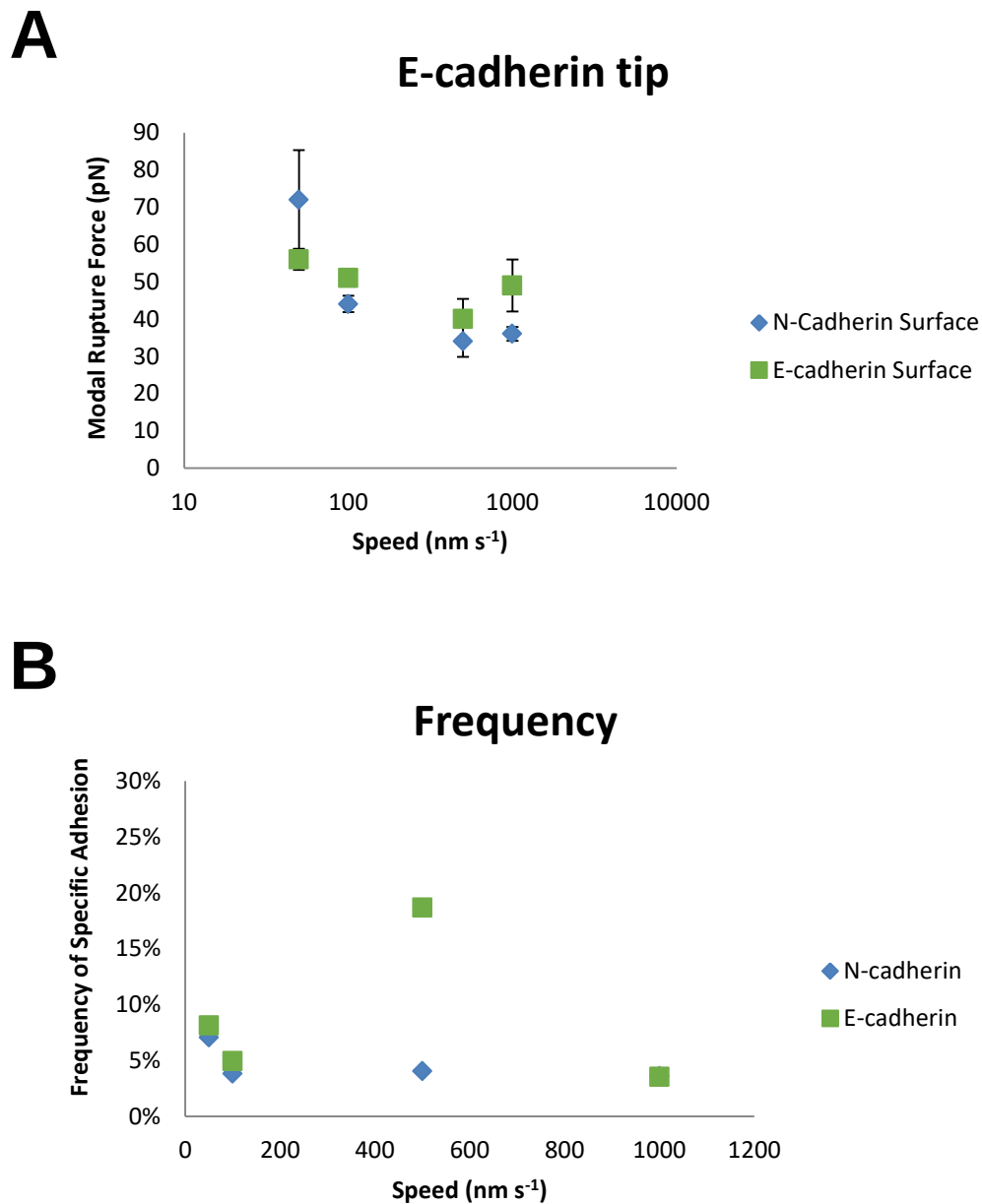


**Figure 3.19. Heterophillic interactions measured with an E-cadherin functionalized AFM tip.** The AFM tip was brought into contact with a sample surfaces functionalized with either E- or N-cadherin to study heterophillic binding between cadherin ectodomains. (A) Force measurements were carried out at four different tip speeds. ( $1 \mu\text{m s}^{-1}$ ,  $500 \text{ nm s}^{-1}$ ,  $100 \text{ nm s}^{-1}$ ,  $50 \text{ nm s}^{-1}$ ). Tip-surface interactions were measured and recorded 500 times at three different positions over a distance of 500 nm from the surface for one AFM tip. Data shown represent the specific bond rupture force values measured in the experiment. Rupture forces of homophillic and heterophillic interactions decreased with increasing tip velocity. Error bars shown represent the standard deviation of the modal value of recorded forces. (B) The binding frequencies of homophillic and heterophillic interactions were in general not strongly affected by changes in tip speed and stayed between 3% to 19%.

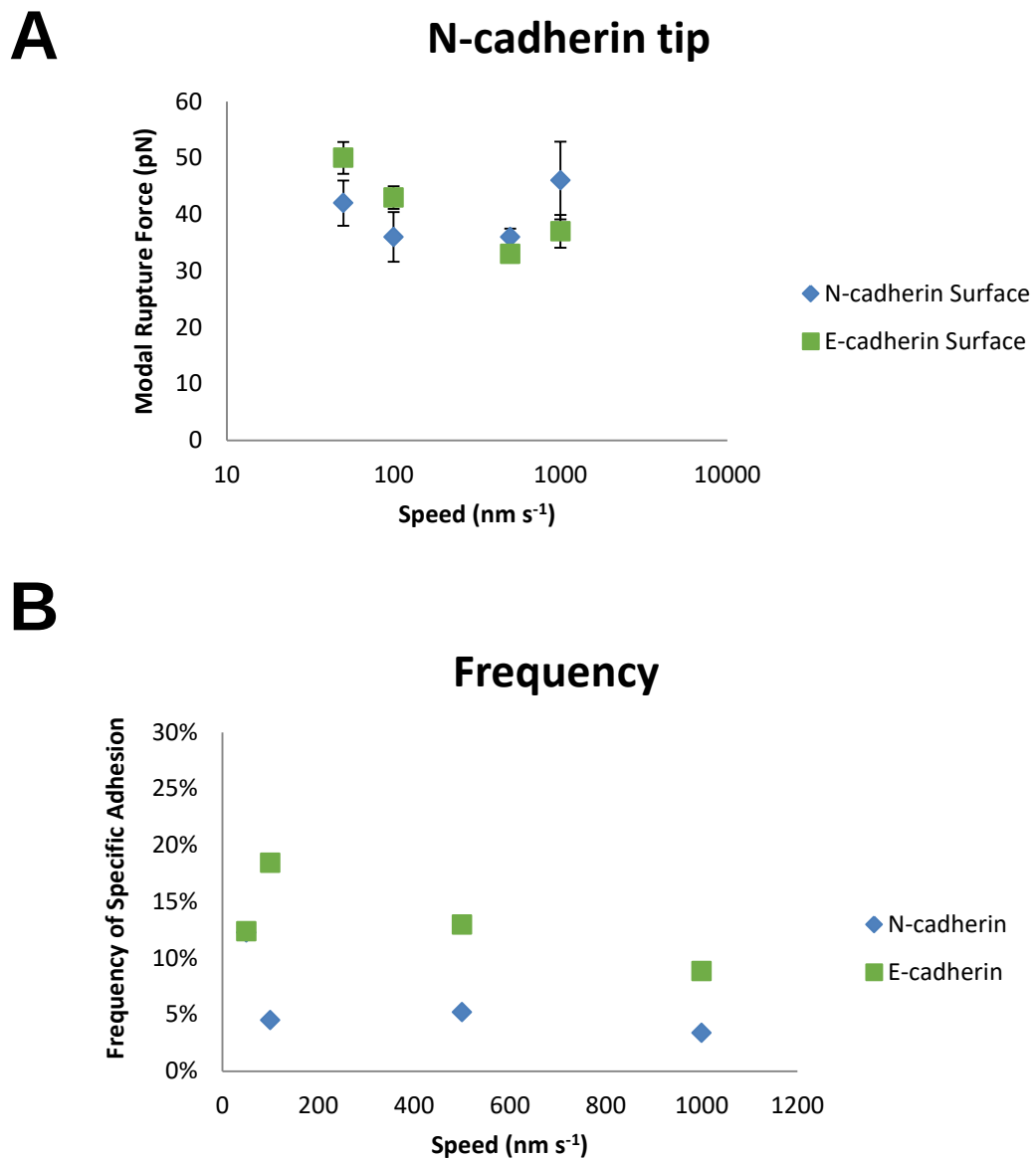


**Figure 3.20.** Heterophillic interactions using an N-cadherin AFM tip. The AFM tip functionalized with N-cadherin was brought into contact with a sample surface functionalized with either E- or N-cadherin to study heterophillic binding between cadherin ectodomains. (A) Force measurements were carried out at four different tip speeds. ( $1 \mu\text{m s}^{-1}$ ,  $500 \text{ nm s}^{-1}$ ,  $100 \text{ nm s}^{-1}$ ,  $50 \text{ nm s}^{-1}$ ). Tip-surface interactions were measured and recorded 500 times at three different positions over a distance of 500 nm from the cell surface for one AFM tip. Data shown represent the specific bond rupture force values measured in the experiment. Rupture forces of homophillic and heterophillic interactions decreased with increasing tip speed. Error bars shown represent the standard deviation of the modal value of recorded forces. (B) The binding frequencies of homophillic and heterophillic interactions were in general not affected by changes in tip speed.

The frequency for specific binding events was in general not affected by changes in the tip speed and remained within the range of 3% to 18% (Fig.3.19.B)(Fig.3.20.B). The observation that heterophilic bonds formed between E-cadherin and N-cadherin ectodomains and the strong similarity in their rupture forces and speed dependence was not surprising, given the strong similarity in structure and function of these classical type I cadherins<sup>130</sup>. Furthermore the results were in agreement with publications that suggest that cadherins are not exclusively specific mediators of cell adhesion<sup>132, 133</sup>. These findings build on the established assumption that cadherins can solely interact in a homophilic way to determine cell sorting and recognition. This initial understanding of cadherin interactions was based on early cell aggregation assays in which cells segregated according to the type and quantitative expression level of their cell adhesion molecules (CAMs)<sup>10, 130</sup>. Ultimately the mechanisms that underlie cell aggregation and segregation are not yet fully understood and recent studies that investigate the morphogenetic roles of cadherin aim to explain interactions of cadherin as an intricate combination between biophysical properties of the ectodomain, linkage to the cytoskeleton and intracellular signalling cascades mediated by the intracellular domain of cadherin rather than solely as homophilic mediators of cell adhesion<sup>134</sup>.

### 3.4. Conclusions

The single molecule force spectroscopy experiments could demonstrate that the surface functionalization technique used to attach E- and N-cadherin ectodomains to AFM tips and silicon wafers could produce single cadherin bonds and that homophilic and heterophilic bond rupture forces of cadherins could be measured, as the values obtained for these detachment forces were within the range of rupture forces reported in literature<sup>12, 15, 117, 118</sup>.

Rupture forces for homophilic and heterophilic bonds between the E and N cadherin ectodomains decreased with increasing tip speeds and had modal rupture forces ranging from 20 pN to 80 pN. Molecular bonds that formed between cadherin ectodomains were predominantly single bond interactions according to Poisson statistics<sup>85, 86</sup>. When brought into contact with one another heterophilic adhesion between E-and N-cadherin could be observed. These bonds had similar rupture forces and binding frequencies compare to homophilic bonds. No substantial differences could be observed between E-cadherin or N-cadherin homophilic interactions on functionalized surfaces in response to externally applied force or in their binding frequency.

The specificity of cadherin interactions could be demonstrated by adding binding inhibitors such as EGTA and cadherin specific antibodies.

By having developed a method to study interactions of E- and N- cadherin at the single molecule level it was now possible to study the interactions of cadherins on the surface of undifferentiated mouse embryonic stem cells (mES).

# **Chapter IV**

## **Neuronal differentiation of embryonic stem cells**

### **4.1. Introduction**

With the methodology in place to study the interactions of E-and N-cadherin at the single molecule level using AFM force measurements, the study of such interactions with native cadherin on undifferentiated mouse embryonic stem cells (mES) and neuronal precursor (NP) cells was now possible, in theory. However, first it was necessary to show the presence of both E-and N-cadherin on both of these cell types.

To this end, immunohistochemistry (IHC) staining was applied to label E- and N-cadherin on undifferentiated mES cells and progenitor cells of different stages along the neuronal differentiation pathway to show the changes in cadherin expression. This staining technique relies on the recognition of cadherin by a primary antibody followed by the recognition of the primary antibody by a secondary antibody labelled with a fluorophore <sup>114, 115</sup>.

Previous studies have linked the expression of E-cadherin to the pluripotent state of embryonic stem cells <sup>46-55</sup>. This was demonstrated by genetic deletion studies in which a loss of E-cadherin caused mES cells to differentiate and lose the expression of specific markers of pluripotency, such as the four Yamanaka transcription factors (OCT4, SOX2, KLF4 and c-MYC) <sup>141</sup> and the stage-specific embryonic antigen 1 (SSEA-1) <sup>42</sup>.

N-cadherin expression on the other hand is known to mediate differentiation, migration and epithelial-mesenchymal transitions during embryogenesis<sup>135</sup>.

Another important function of N-cadherin is to assist in the maintenance and development of the vertebrate nervous system. N-cadherin has been implicated in the formation of the neural tube during neurulation and to facilitate adhesion between neuronal synapses and axons<sup>136, 137</sup>.

In the context of this study the expression of N- cadherin therefore served as indicator for 1) the loss of pluripotency and 2) the differentiation towards a defined neuronal lineage with distinct phenotypic characteristics. By using IHC staining for either E- or N-cadherin we could demonstrate a change in the expression level of these cadherins on undifferentiated mES cells, on progenitor cells at different stages along the neuronal differentiation pathway and on fully differentiated precursors of the neuronal lineage (NP cells).

## **4.2. Materials and Methods**

### **4.2.1. Mouse embryonic stem cell (mES) culture and maintenance of pluripotency**

mES cells that were derived from the inner cell mass of the mouse blastocyst (cell line CGR8) were directly seeded onto tissue culture flasks coated with 2% gelatin (Sigma-Aldrich) and maintained according to the SOP developed within the division of regenerative medicine and tissue engineering (School of Pharmacy, University of Nottingham 28/03/14). In order to maintain their undifferentiated state, elevated concentrations (103 Units/ml) of leukaemia inhibitory factor (LIF) (Calbiochem) were added to the culture medium, a method known as feeder free mES culture, as this technique does not rely on

a fibroblast based feeder layer <sup>138-140</sup>. mES cells chosen for subsequent experiments had a cell passage number that was lower than 25, as it had been previously observed that mES cell lines at high passage numbers experience alterations in morphology, response to stimuli, growth rates, protein expression and transfection efficiency, compared to lower passage cells <sup>141-146</sup>.

#### **4.2.2. *In Vitro* generation of NP cells from mES cells**

mES cells were differentiated into a neuronal lineage following a procedure published by Bibel *et al.* and adjusted in collaboration Dr. Omar Hamid (University of Nottingham), to generate homogenous and defined populations of neurons from undifferentiated mES cells <sup>147</sup>. This procedure consisted of four essential steps (Fig.4.1.). First, mES cells were suspended in 10 mL differentiation media (Serum free, DMEM F12, Neurobasal media, 6-mercaptoethanol, L-glutamine, Penicillin, N2 Supplement, B27 Supplement (Thermofisher Scientific)) in 10 cm diameter non adherent cell culture dishes (Thermofisher Scientific) and incubated at 37°C, 95% relative humidity and a CO<sub>2</sub> level of 5 % for two days in order to induce neuronal differentiation and embryoid body formation (Fig.4.1.A).

Embryoid bodies (EB's) are 3-dimensional aggregates of mouse embryonic stem cells that spontaneously form when embryonic stem cells are cultured in the absence of anti-differentiation factors and on non-tissue culture-treated dishes to prevent attachment <sup>148-150</sup>. Embryoid bodies were allowed to form and were collected after two days. The EB's were resuspended in differentiation media that contained 1 µM retinoic acid (Sigma-Aldrich)

(Fig.4.1.B). After three days of incubation the embryoid bodies were collected and placed into differentiation media without retinoic acid for 24 hours. In the final step embryoid bodies were dissociated and plated on poly-L-ornithine (Sigma-Aldrich) and laminin (Sigma-Aldrich) coated cell culture plates in a cell density of  $1.5 \times 10^5 \text{ cm}^2$ . After two days most precursor cells showed spindle shape morphology and displayed many additional structural characteristics of the neuronal phenotype such as elongated axons, cell bodies and interconnected dendrites (Fig.4.1.D) <sup>45</sup>.

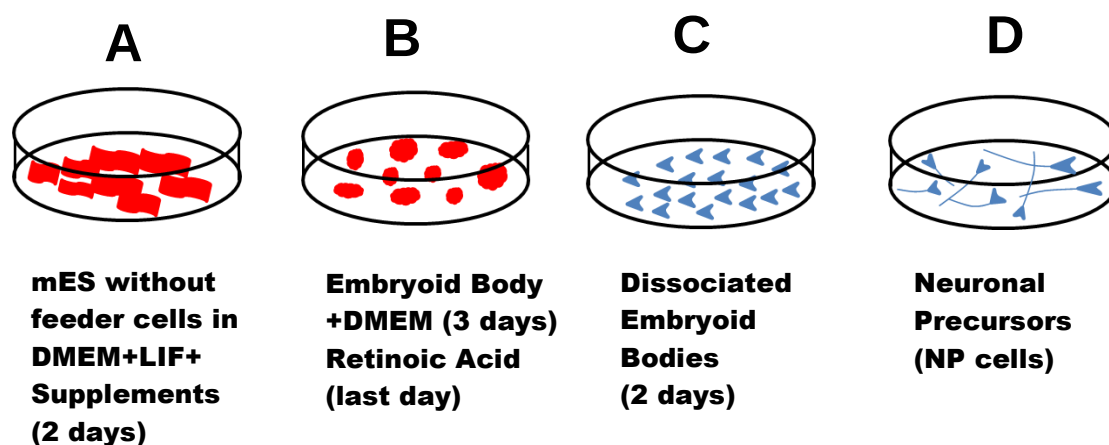


Figure 4.1. Schematic representation of the neuronal differentiation procedure in feeder free cell culture <sup>147</sup> (A) Flat monolayer of undifferentiated mES cells. Cells were grown on a gelatin coated surface, in the presence of LIF for 2 days. (B) mES cells in non-adherent cell culture dishes formed aggregates (embryoid bodies (EB's)). After 3 days of embryoid body formation retinoic acid was added and the EB's were incubated for one more day. (C) EB's were dissociated after four days and plated onto poly-L-ornithine and laminin coated cell culture plates in differentiation media. (D) After 48 hours most precursor cells had spindle shape morphology.

### 4.2.3. Immunohistochemistry staining (IHC)

In order to determine the presence of E- and N-cadherin on the surface of mES cells, immunohistochemistry staining (IHC) was employed in order to label these proteins on the cell surface. Figure 4.2. illustrates the principle of this staining technique <sup>45</sup>. First cadherins on the cell surface are recognized

by a primary antibody that is specific for a particular type of cadherin. The primary antibody, in turn, is recognized by a secondary antibody that binds specifically to the primary antibody. The Fc region of the secondary antibody is labelled with a fluorophore that can re-emit light upon light excitation.

Cells were fixed at specific stages in their development using 3.7 % paraformaldehyde (Sigma –Aldrich) in PBS (Fisher Scientific). In order to label the cadherin on the cell surface, cells were permeabilised with Triton X for 30 to 40 minutes, washed in PBS and blocked with 3% donkey serum in PBS (Sigma) for 30 minutes. After removing the blocking solution the primary antibody specific for either E- or N-cadherin (E-cadherin: polyclonal goat IgG (R&D Systems); N-cadherin: polyclonal sheep IgG (R&D Systems)) was added and the cell samples were incubated over night at 4°C. The primary antibody solution was then removed through washing the sample in PBS. The secondary antibody solution was then added (E-cadherin: Alexa Fluor 546 donkey anti-goat IgG. (Invitrogen); N-cadherin: Northern Lights anti sheep IgG-NL557. (R&D Systems)), labelling the cell samples with a red fluorescent dye. The cells were then incubated for two hours at room temperature. Appropriate controls without or with only the primary or secondary antibody were performed to ensure that the E- and N-cadherin stain was binding specifically (data not shown). The excess secondary antibody was then removed by washing the cell samples with PBS and the Hoechst 33258 solution (Sigma-Aldrich) in water (1 mg/mL) was added. The Hoechst stain labels the DNA inside the nucleus with a blue fluorescent dye and served here as a control and reference to the cadherin stain <sup>151</sup>. Furthermore the Hoechst

stain helped to distinguish individual cells in the immuno stained images (see Section 4.3.2. below).

The samples were incubated for 15 minutes at room temperature and the Hoechst solution was removed. The samples were then washed in PBS prior to fluorescence imaging.

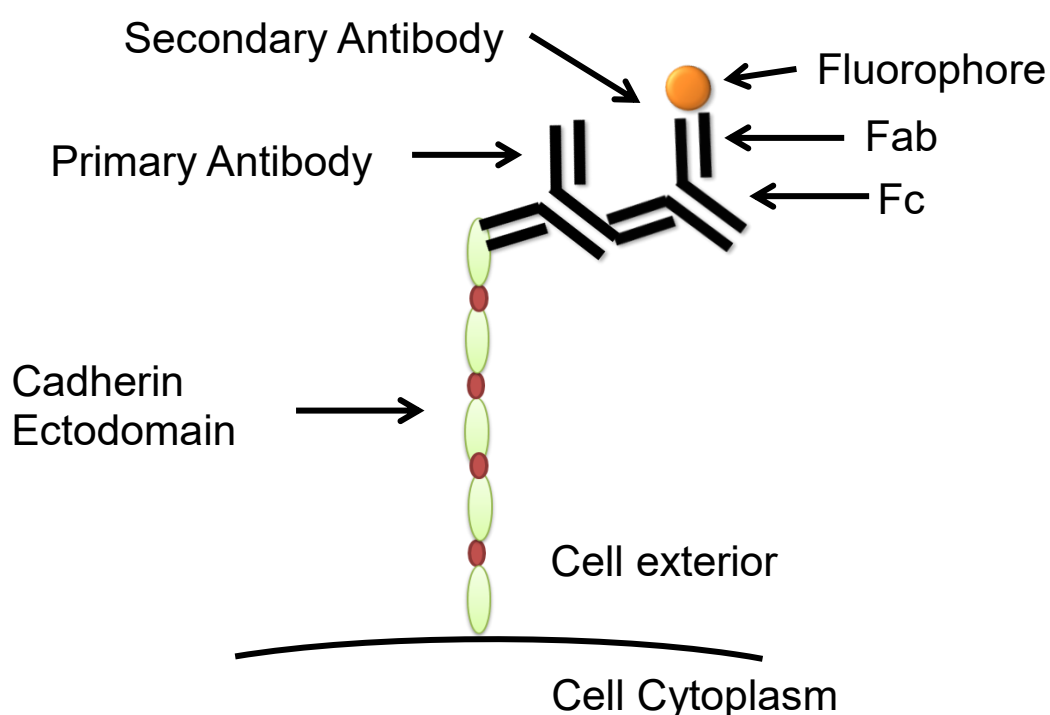


Figure 4.2. Schematic representation of the IHC staining procedure <sup>45</sup>. First the cadherin molecule is recognized by an antibody specific for the cadherin then the fluorescence labelled secondary antibody binds to the attached primary antibody. This allows the labelling of E- and N-cadherin on the cell surface of mES cells and neuronal precursors.

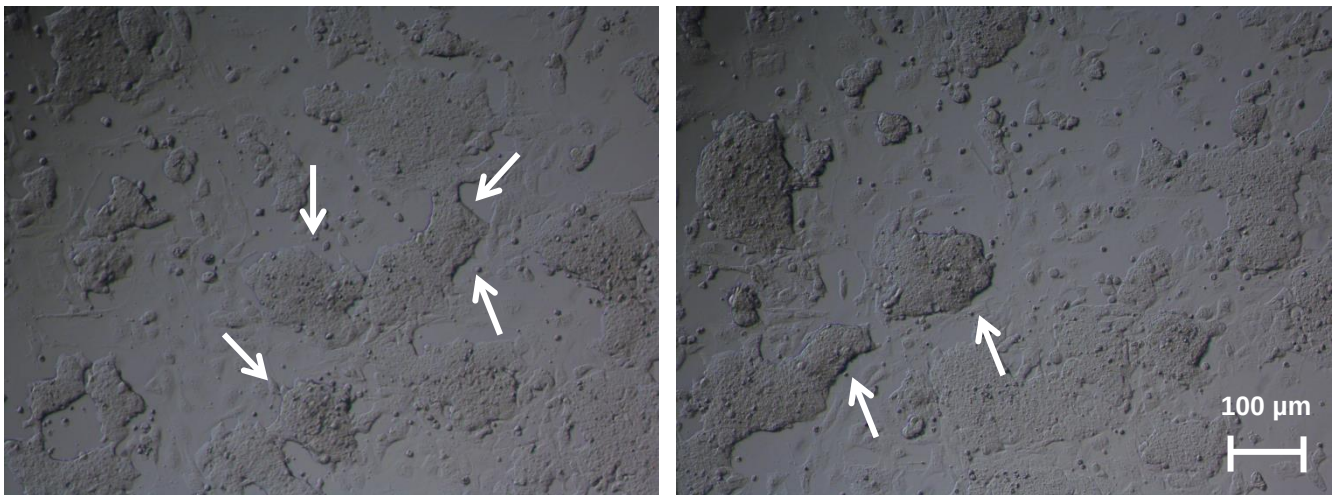
## 4.3. Results and Discussion

### 4.3.1. Differentiation of mES into NP cells

mES cells were differentiated based on the procedures described above (Section 4.2.2.). The phase contrast image in Figure 4.3.A shows undifferentiated mES cells in the presence of LIF, possessing the desired

phenotype with a flat, spread out appearance with defined sharp edges which are indicators of their pluripotency<sup>147</sup>. Thus, these cells were selected for IHC staining and subsequently to form EB's and for neuronal differentiation. Terminal differentiated NP cells could be observed after eight days of differentiation and showed many structural features of fully differentiated neurons such as cell bodies, axons and dendrites (Fig.4.3.B.)<sup>4</sup>.

**A**



**B**

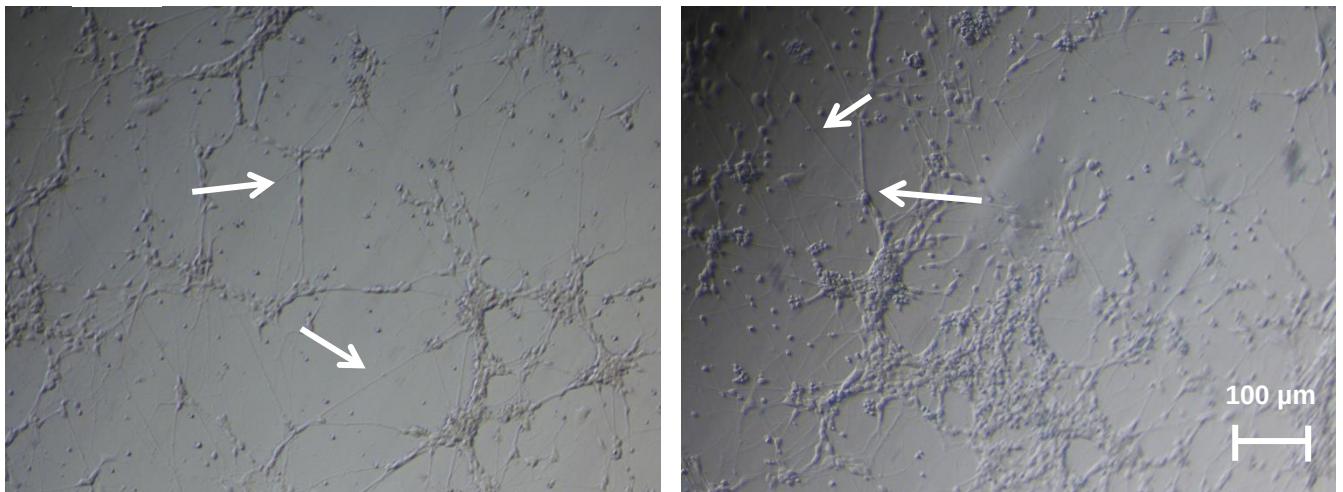


**Figure 4.3.** Phase contrast images of mES cells and NP cells. (A) Phase contrast images of undifferentiated mES cells in feeder free cell culture. Cells showed defined sharp edges (as indicated by the white arrows) and formed an overall flat monolayer of around 60% to 70% confluency. The smaller structures between the cells are connective tissue and developing cells. (B) NP cells derived from undifferentiated mouse embryonic stem cells in feeder free cell culture. mES cells differentiated into a homogenous population of NP cells and displayed structural characteristics of the neuronal phenotype such as elongated axons, cell bodies and interconnected dendrites (As indicated by the white arrows). The images shown are taken from the same sample for mES and neurons respectively.

### **4.3.2. Cadherin expression on mES and NP cells**

In order to show cadherin expression during differentiation of mES cells and detect potential changes in cadherin expression, mES cells were fixed at specific stages during their development towards NP cells. Once the cells were fixed, immunohistochemistry staining was used to detect E-or N-cadherin expression using cadherin specific antibodies.

To assess the cadherin expression in the early stages of neuronal differentiation, mES cells were treated with LIF for four days to maintain them in their undifferentiated state (Fig.4.4.A) and compared to those cultured in the absence of LIF for four days (Fig.4.4.B). Figure 4.4.A shows the distinct morphology of undifferentiated feeder free mES cells. Again, they have a spread out appearance forming a characteristic, flat monolayer of cell colonies with defined sharp edges (see Fig.4.3.A above). The morphology of mES cells was found to be crucial for the selection of uncommitted mES cells, as it has been observed that despite being in the presence of LIF, over confluent mES cells also have a tendency to differentiate, and form embryoid body (EB) like structures that are attached to the culture dish bottom and consist of differentiated cell types committed to various different differentiation pathways<sup>31</sup>. In order to select for undifferentiated mES cells the phase-contrast image appearances of the mES cells and their degree of confluence were carefully monitored before selecting them for EB formation and neuronal differentiation.

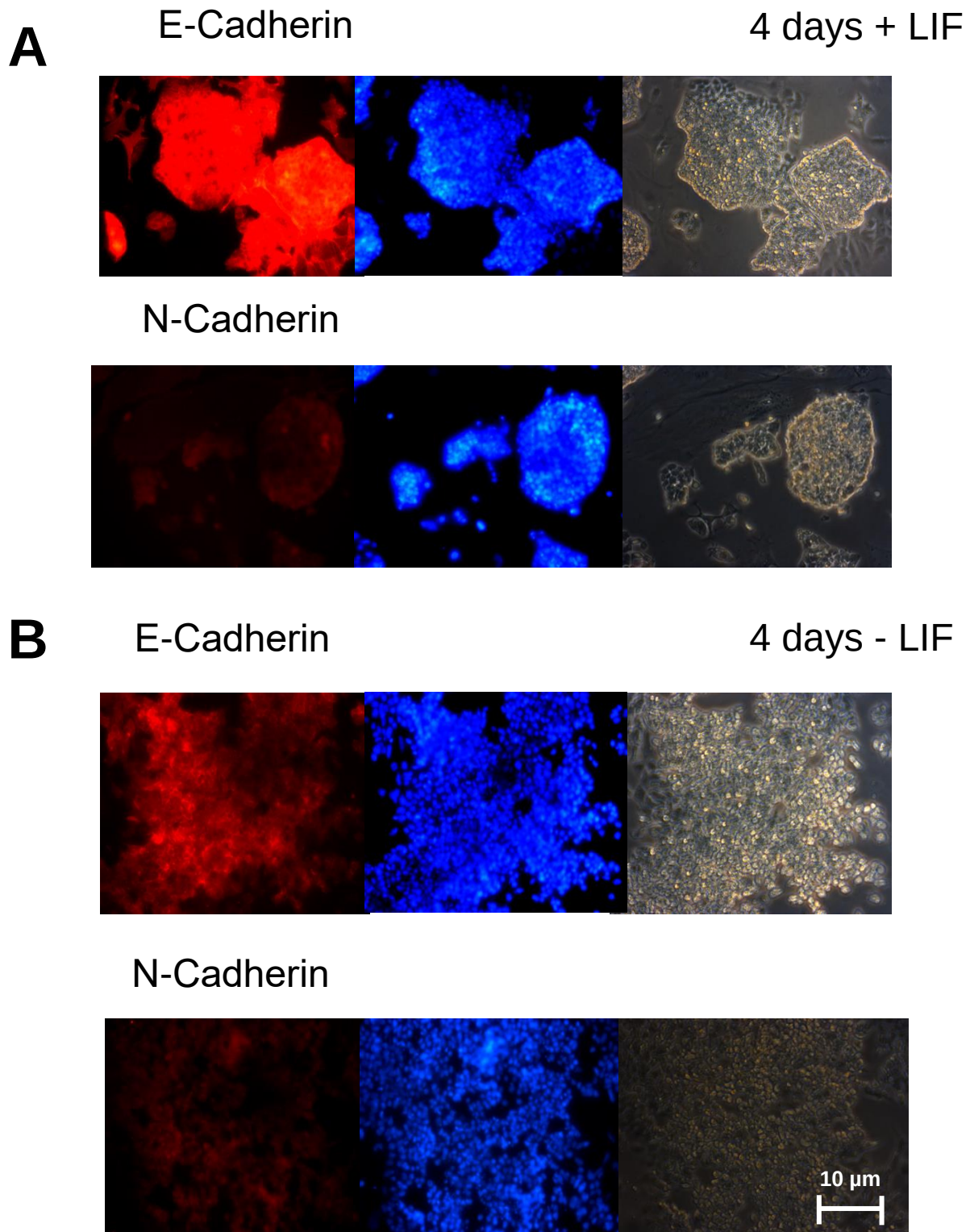


Figure 4.4. Fluorescence images of IHC stained mES cells. The images on the left show the antibody stain for E-or N-cadherin in red. The images in the middle show the Hoechst stain of the DNA inside the nucleus in blue. The images on the right show the phase contrast of the image. (A) mES cells were treated with LIF for four days to maintain their undifferentiated state or (B) cultured in the absence of LIF for four days in order to simulate the early stages of neuronal development.

Furthermore it can be seen in Figure 4.4.A that the fluorescence intensity of the E-cadherin stain is higher compared to that of N-cadherin (with or without LIF), indicating a higher cell surface expression level of E-cadherin compared to N-cadherin. This provided more evidence for the pluripotency of these mES cells as E-cadherin is a known indicator for pluripotency <sup>40, 152</sup>. The images of Figure 4.4.A correspond to the step “A” in Figure 4.1. which details the differentiation procedure steps for generating NP cells in the methods section. In order to study cadherin expression in the early stages of embryonic differentiation, cells were also cultured without LIF for 4 days. Figure 4.4.B shows the appearance of cells cultured in this way. It can be seen that the flat, spread out appearance and defined sharp edges of cells cultured in the presence of LIF, is absent in these cells. Instead, they had no defined edges and were growing on top of each other in multiple layers. The fluorescence intensity for E-cadherin decreased compared to cells cultured in the presence of LIF, suggesting that these cells had undergone differentiation and had lost their pluripotency.

To investigate cadherin expression levels during later stages of neuronal differentiation, cells were fixed when they had formed embryoid bodies (EB's). Figure 4.5.A shows the appearance of mES cells when they had formed these structures after being grown in the absence of LIF, on non-tissue culture-treated dishes to prevent attachment. Cells formed 3 dimensional aggregates that were freely floating in the cell culture medium.

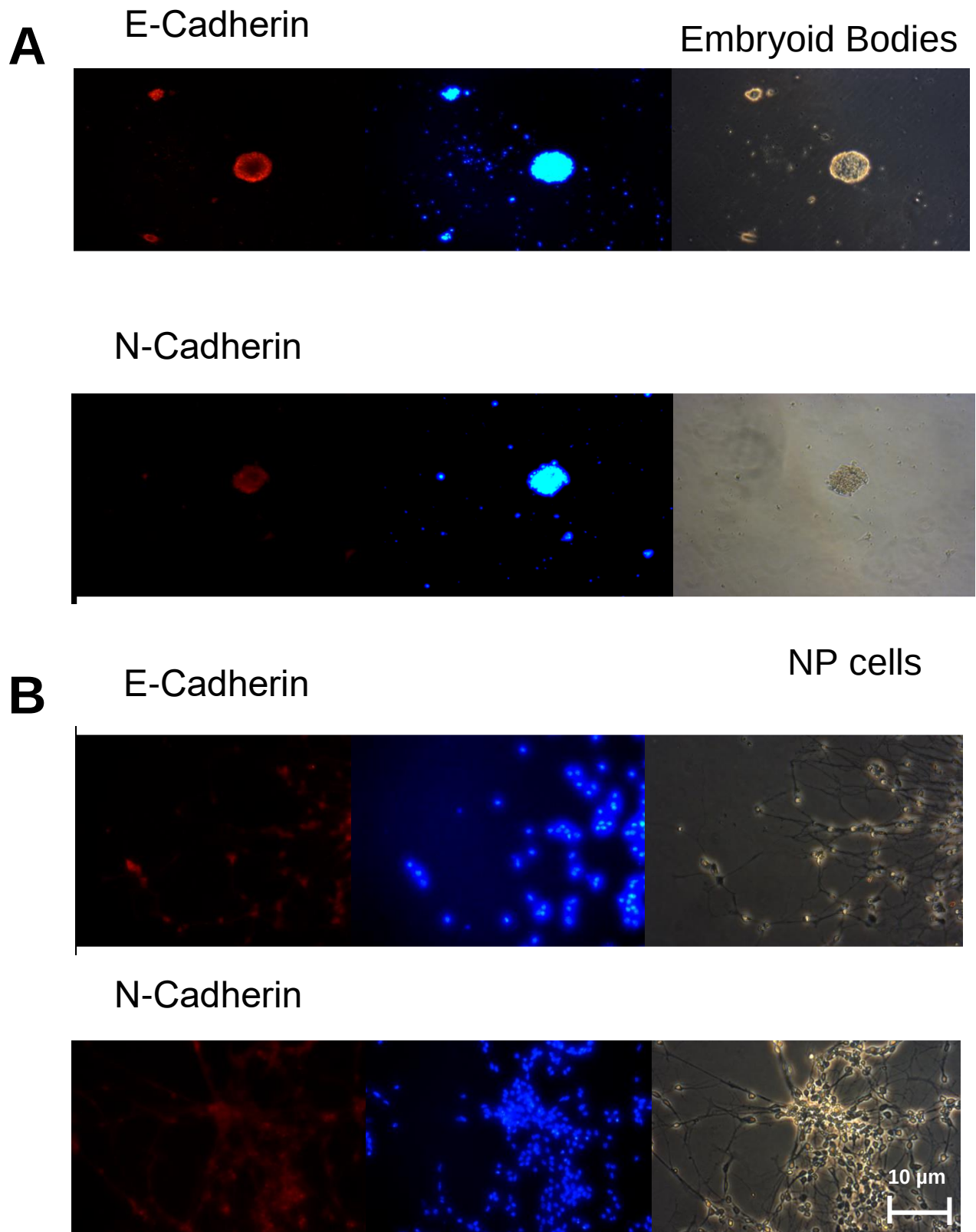


Figure 4.5. IHC staining of embryoid bodies (EB's) and neuronal precursor (NP) cells for E- and N- cadherin. The images on the left show the antibody stain for E-or N-cadherin in red. The images in the middle show the Hoechst stain of the DNA inside the nucleus in blue. The images on the right show the phase contrast of the image. (A) EB's formed after three days of cell culture in the absence of LIF on untreated non adherent cell culture plates. (B) NP cells derived from undifferentiated mES cells after eight days of feeder free cell culture. The fluorescence signal of the E- cadherin stain relative to the mES stain shows a marked reduction as embryoid bodies were dissociated to form NP cells.

This stage, showing EB formation, refers to step B in Figure 4.1. Highly differentiated NP cells could be observed after eight days of feeder free cell culture. Figure 4.5.B shows the appearance of NP cells showing many features of the neuronal phenotype such as cell bodies, axons and dendrites. In order to estimate the quantitative difference in fluorescence intensity observed in these images, the ratio of the mean fluorescence signal of the secondary antibody (shown in red) and the mean fluorescence signal of the DNA stain, Hoechst (shown in blue), was determined for each differentiation stage. Figure 4.6. shows the ratio values for E- and N-cadherin at the different development stages under consideration. The fluorescence signal for E cadherin was high in undifferentiated mES cells and decreased relative to this stage when cells were cultured in the absence of LIF for four days. This trend continued as mES cells were allowed to form EB's and after induced neuronal differentiation.

N-cadherin staining showed low fluorescence intensities during the undifferentiated stage of mES cells, in the presence of LIF, as well as in the absence of LIF and during EB formation. Only after induced neuronal differentiation did the antibody-DNA label ratio increase and exceeded that of E-cadherin (Fig.4.6.). Due to a limited number of repeats of this experiment the data of these results must be considered with caution and more investigation is required to validate the observed trends presented here.

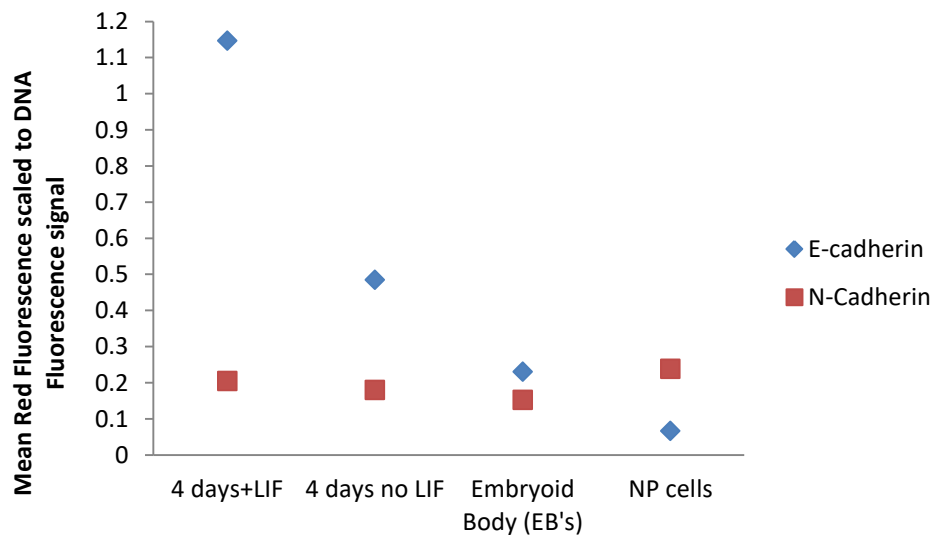


**Figure 4.6. IHC stain: Relative cadherin expression analysis.** Fluorescence image brightness means presented here as ratio between red fluorescence (Antibody specific for E-or N-cadherin) and blue fluorescence (Hoechst stain of DNA in the nucleus) in the same image. This ratio value of E-cadherin was high on undifferentiated mES cells compared to N-cadherin, but decreased with each observed stage towards NP cells. On NP cells the N-cadherin red to blue ratio was higher than that of E-cadherin.

The results of the IHC stain demonstrated not only that E- and N-cadherin are expressed on undifferentiated mES cells and the intermediate neuronal development stages under consideration but also that the expression levels of these proteins changed with progression towards neuronal differentiation. This change of cadherin expression is consistent with studies that have demonstrated the importance of E- and N-cadherin in facilitating differentiation during embryonic developmental processes, and how cadherins mediate the formation of various tissue types during gastrulation and neurulation <sup>153-155</sup>. For example homophilic E-cadherin interaction binds cells firmly together during compaction and enables subsequent cellular differentiation during the cleavage phase in which cells divide to form different parts of the early embryo <sup>155</sup>. N-cadherin interactions can be observed during the formation of

the neural tube during neurulation in which they help cells to separate from the overlying ectoderm<sup>4</sup>. When viewed as a function of the time elapsed after omission of LIF, and the concomitant loss of pluripotency of mES cells, the change in expression level of E- and N- cadherin followed a reciprocal trend meaning that once pluripotency was lost E-cadherin expression was reduced whereas N-cadherin expression remained at constant low values and only picked up days after loss of pluripotency (Fig.4.6.).

This trend in E-and N-cadherin expression was also observed by D'Amour *et al.* who presented a reciprocal expression kinetic of expression levels of E- and N-cadherin mRNA and proteins five days after induced definitive endoderm formation in differentiating human embryonic stem cell (hES) cultures<sup>156</sup>. Initially E-cadherin is predominantly expressed and helps to maintain the pluripotency of embryonic stem cells and to maintain stable attachments and positions of these cells<sup>40, 156</sup>. Once differentiation during embryonic development, e.g. via epithelial mesenchymal transition, is initiated other types of cadherin and cell adhesion molecules (CAMs) such as N-cadherin<sup>4</sup> and Integrin CD29<sup>31</sup> are expressed on the cell surface to allow cells to migrate and form new types of tissue and at the same time expression of E-cadherin is gradually reduced<sup>4, 156</sup>.

Although the fluorescence ratio results (Fig.4.6.) indicated a change in the expression level of E-and N-cadherin they can only serve here to capture general trends in the expression of these proteins. Only by monitoring the expression of specific marker genes and proteins such as SSEA (1,3,4)<sup>40</sup>, E-cadherin<sup>46-55</sup> and CD 9<sup>157</sup> for pluripotent mES cells and NeuN<sup>158</sup>, MAP-2<sup>159</sup>, and NSE<sup>160</sup> for terminally differentiated non mitotic neurons would it be

possible to ascertain their differentiation state. Thus further investigation is required to capture the extent of change in cadherin expression according to the cell differentiation stage under observation. Due to time constraints no alternative approaches to verify the differentiation state of mES cells or NP cells could be carried out. Such alternative approaches could include the use of quantitative polymerase chain reaction (qPCR), Western or Northern blot, microarray analysis and fluorescence in situ hybridization (FISH). However, for the purpose of this study the demonstration of the presence of E- and N-cadherin and a change in their relative expression level on the cell surface during the differentiation stages observed was sufficient to investigate how phenotypic changes during neuronal differentiation influence biophysical properties of E- and N-cadherin bonds and their response to applied forces.

#### **4.4. Conclusions**

In summary, it was possible to demonstrate a procedure that can induce the differentiation of mouse embryonic stem cells into a distinct neuronal lineage through the addition of retinoic acid and a defined neuronal differentiation medium. The presence of E- and N cadherin could be demonstrated on both undifferentiated mES cells and NP cells that possessed all the structural features of fully differentiated neurons such as elongated axons, cell bodies and interconnected dendrites. Furthermore by labelling E- and N-cadherin on the cell surface as well as labelling the DNA inside the nucleus it was possible to compare the ratio of fluorescence intensity of cadherin relative to the DNA stain (Hoechst). It could be shown that the fluorescence intensity for E-

cadherin underwent a marked decline through the progression of differentiation stages from undifferentiated mES cells towards NP cells. N-cadherin levels did not display such trends and increased to a small degree in end stage NP cells.

Having demonstrated the presence and change in expression level of E- and N-cadherin on the surface of undifferentiated mES cells and differentiated NP cells we could now apply the insights we had gained about the study of cadherin interactions at the single molecule level using AFM, to the study of cadherin interactions on these cell types.

# **Chapter V**

## **Cadherin interactions probed on mES cells by AFM force measurements**

### **5.1. Introduction**

After demonstrating the presence and change in expression level of E -and N-cadherin on undifferentiated mES cells and NP cells, the interaction of cadherins on these cell types could now be investigated using AFM. Although previous molecular and genetic approaches have identified cadherins as adhesion proteins and provided insight into their ligand specificities and the processes in which they are involved <sup>5, 10</sup>, the exact molecular mechanisms by which cadherins regulate adhesion in early embryonic development are still an open debate. To understand cadherin mediated cell adhesion, therefore, the vast amount of qualitative data that is available must be augmented with quantitative data of the physics of cadherin bonds <sup>161</sup>. Previous studies that have investigated the biophysical nature of cadherin interactions on living cells have used methods such as flow <sup>162</sup>, cell aggregation and spinning disc <sup>163</sup> assays. The shear force that is exerted on cells in these assays depends on parameters such as media flow, cell size, cell shape and how the cell is attached to the substrate and can only yield estimations of global cell to cell adhesions.

For a more quantitative and controlled approach to investigate cadherin bond formations, single-cell based methods are thus required <sup>164</sup>. For the work of this thesis, therefore, a set-up was used that measured the contributions of individual cadherin bonds to cell adhesion rather than observing average behaviour of global cell to cell interactions. To this end, AFM tips were functionalized with cadherin ectodomains (as described in Chapter III, Section 3.2.2.1.) and their interaction with native cadherin expressed on the surface of undifferentiated mES cells, as well as NP cells was investigated using AFM based force measurements.

Panchoran *et al.* <sup>165</sup> used a variation of this set up in which cadherin expressing cells were attached on the AFM cantilever and brought into contact with target cells on a substrate surface. By applying AFM based dynamic force spectroscopy they investigated interactions between E- and N-cadherins expressed on the surface of living chinese hamster ovary (CHO) cells. They found that E- and N-cadherin bonds displayed quantitative differences in their bond rupture forces. Homophillic N-cadherin bonds showed low mean detachment forces (30 pN and 40 pN) at loading rates of 100 pN s<sup>-1</sup> and 10,000 pN s<sup>-1</sup>, respectively, with a single-barrier energy potential to bond dissociation. In contrast to E-cadherin homophillic bonds displayed high bond rupture forces (73 pN and 157 pN) at 100 pN s<sup>-1</sup> and 10,000 pN s<sup>-1</sup>, respectively, with two barriers in the energy landscape to bond dissociation. The differences in bond properties were attributed by the authors to the different functions that are associated with E- and N-cadherin in mediating cell adhesion.

They hypothesized that N-cadherin must form bonds that are less resistant to bond rupture under physiological strain, thereby assisting cells when they need to break away from their neighbours in tissues that are undergoing morphological changes, such as during epithelial mesenchymal transitions <sup>10</sup>. E-cadherin on the other hand forms one bond that has a high dissociation rate, which is quickly formed and released and might aid in rapid cell recognition processes, such as those that occur during cell sorting in developing tissues and another more stable, longer lasting bond, that might be used by E-cadherin once a suitable binding partner is found.

More recently, Fichtner *et al.* immobilized E-cadherin ectodomains (EC1-EC5) on gold surfaces and assessed the adhesive capabilities of these ectodomains by approaching them with L-cells, expressing E-cadherin, with a single L-cell being attached to an AFM cantilever. L-cells are murine fibroblasts which normally lack endogenous cadherin expression <sup>12</sup>. By transfecting L-cells with the appropriate vector to make them express cadherin on the cell surface, cadherin interactions between E-cadherin expressed on the cell surface and recombinant E-cadherin constructs on the gold surface could be investigated. During contact individual cadherin bonds formed and then ruptured when the cell was withdrawn. Furthermore by comparing force spectroscopy data obtained from interactions between two E-cadherin ectodomain constructs (EC1-EC5) to rupture forces of bonds formed between L-cells expressing E-cadherin and their interaction with cadherin constructs on the gold surface, these researchers found that the majority of measured rupture forces had magnitudes that were within the range of single molecule bonds. This suggested that, cell detachment occurred via unbinding

of individual cadherin receptors sequentially rather than simultaneously via the rupture of a large group of cross-linked receptors. The authors hypothesized therefore, that cadherin cell contacts are formed and then reinforced primarily by increasing the number of engaged receptors and then organizing these into loosely associated adhesive clusters.

The insights gained from these experiments help to elucidate the binding mechanism of cadherins, information that is of potential importance in the development of new medical treatments. While previous studies investigating cadherin force measurements on cells have focused on cell types such as epithelial or fibroblasts cell lines (e.g. L-cells and CHO cells respectively) <sup>12, 165</sup>, the interactions of cadherins on undifferentiated embryonic stem cells have, as of yet, have not been explored with cell based force spectroscopy (CFS). This is the goal of this chapter.

If the undifferentiated, pluripotent state of embryonic stem cells, as well as the healthy function of cells belonging to a specific tissue type, could be defined by a “biophysical fingerprint” associated with the measurement of cadherin bond rupture forces, then such an assay could open the door to new avenues of research and disease detection. For this reason the aim of this chapter was to investigate E-and N-cadherin interactions on the surface of undifferentiated mES cells, as well as during an early stage of neuronal differentiation of these cells, to describe the change in cadherin interactions as they progress from one developmental stage to the next. To accomplish this goal, adhesion forces between purified cadherin ectodomains immobilized on AFM tips and native cadherins on mES cells were recorded and compared to adhesion forces on NP cells using AFM based CFS.

## **5.2. Materials and Methods**

### **5.2.1. Acquisition of force measurements on mES cells**

In order to study the interaction of cadherin ectodomains attached to AFM tips with native cadherin on mES cell surfaces, a customized method was developed as part of the work for this thesis. This was based on the protocol that was developed to study cadherin single molecule interactions (SMFS) as detailed in Chapter III, Section 3.2.2.2.

An AFM was fitted with a fluid chamber that allowed measurements to be made in an aqueous environment under near physiological conditions. mES cells or NP cells were seeded onto cell culture dishes (21.29 cm<sup>2</sup> (Fisher Scientific)) until they reached a sufficient level of confluency (~60% to 70%). mES cells were cultured so that they had a cell passage number that was lower than 25 (as detailed in Chapter IV, Section 4.2.1). The cell culture dishes were then added to the AFM fluid chamber and attached to the chamber bottom. Functionalized AFM tips were then brought into and out of contact with the cell surface of mES cells or NP cells.

Figure 5.1. shows the principle of cell based force spectroscopy (CFS). Force measurements were carried out in mES cell culture medium (DMEM, 10% fetal bovine serum, 1% penicillin, 1% L-glutamine, 100 µM 2-mercaptoethanol)(Thermo Fisher Scientific) warmed to 37 °C. AFM measurements were carried out at room temperature and the temperature was not monitored during the experiments. AFM tips were positioned above high density cell clusters using the bright field microscope built into the AFM and the tip-surface interaction was recorded as force distance (FD) curves.

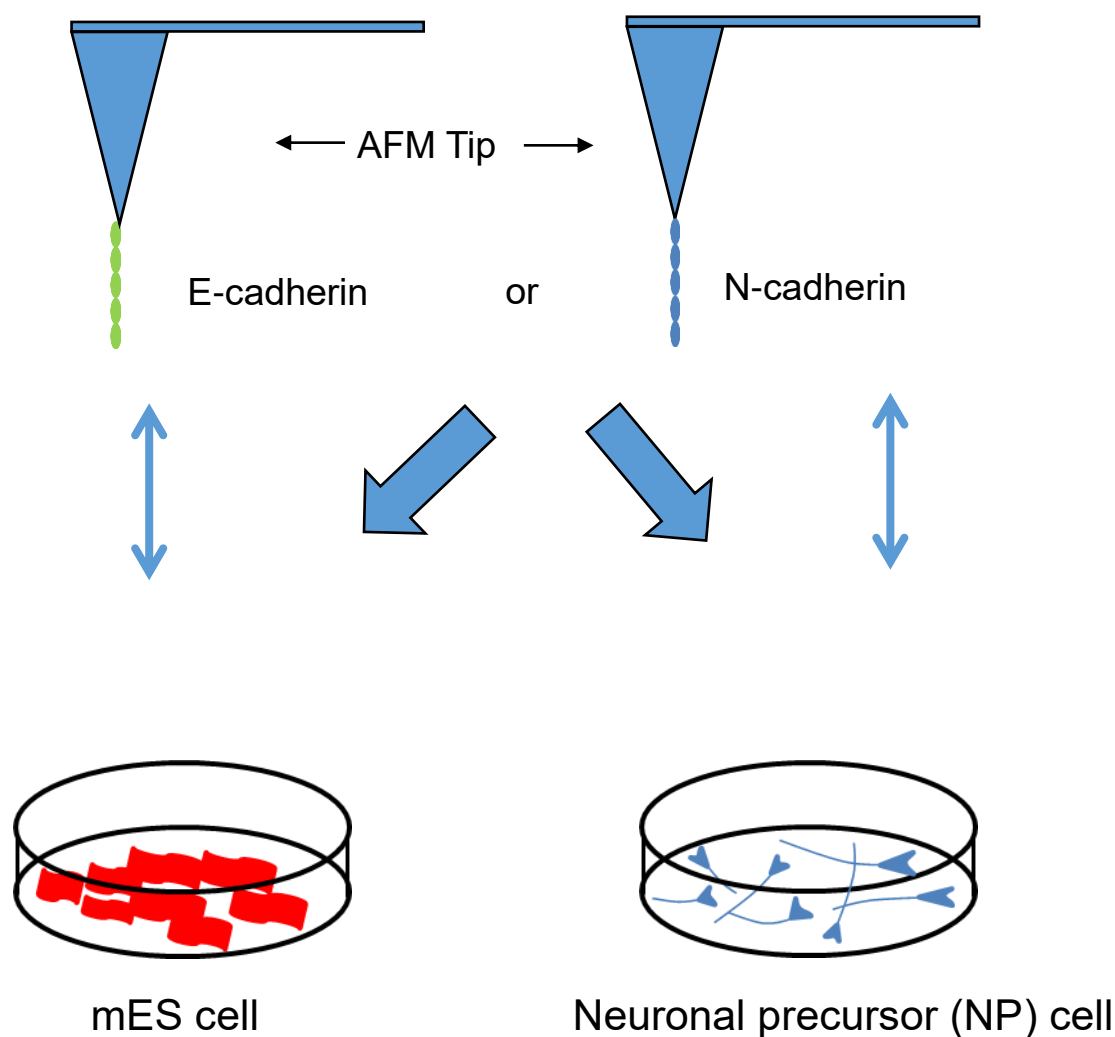


**Figure 5.1. Principle of cell force spectroscopy (CFS) experiments. The ectodomain of either E-or N-cadherin was functionalized to an AFM tip and brought into and out of contact with the surface of undifferentiated mES cells or NP cells. Upon making contact, bonds were formed between the cadherin on the AFM tip and native cadherin on the cell surface. When the AFM tip was retracted, formed bonds ruptured and the AFM tip could be freely removed from the cell surface.**

Figure 5.2. shows the different appearance of undifferentiated mES cells (Figure 5.2.A) and NP cells (Figure 5.2.B) under the AFM cantilever during a force measurement. The flexible surface of the cells meant that the tip had to

move longer distances in CFS compared to SMFS before the set contact force could be reached. The contact force was kept low ( $\sim 100$  pN). Once the AFM had made contact with the cell surface and reached the set contact force, the cantilever had begun to bend and was retracted a result (See Chapter II, Fig.2.1.B). Once the flexibility of the cell membrane was exhausted due to the tip pushing it inwards, it became firm and provided enough resistance for the AFM tip to reach the set contact force. In single molecule experiments a vertical distance of 500 nm travelled by the AFM tip was sufficient to reach the contact force as the AFM tip was pushed against a hard surface (see Chapter III, Section 3.2.2.2.), whereas in cell measurements a maximum distance of 2  $\mu\text{m}$  was chosen to adjust for the stretchable soft surface of the cell membrane. Furthermore because of the flexible cell surface and considering the molecular dimensions of the completely unfolded cadherin ectodomain (270 nm)<sup>120</sup>, specific adhesion events were expected to occur within 300 nm from the cell surface. This maximum distance at which adhesion events were considered specific and selected for data analysis was increased two fold compared to SMFS experiments, again, to adjust for the flexibility of the cell membrane. Because the cell surface is softer than the surfaces used in single molecule experiments in Chapter III it was also not possible to measure interactions of cadherin at the same tip speeds as in SMFS. This was because force measurements carried out at  $50\text{ nm s}^{-1}$  did not yield consistent force distance curves on mES cells. Hence, force measurements were carried out at tip speeds that were adjusted to the cell based experiments (i.e.  $1\text{ }\mu\text{m s}^{-1}$ ,  $750\text{ nm s}^{-1}$ ,  $500\text{ nm s}^{-1}$ ,  $250\text{ nm s}^{-1}$ ). The tip - surface interaction was measured at least 500 times at three different

sample surface positions for each tip speed using the same AFM tip. Data sets were analysed using the procedure detailed in Chapter III, Section 3.2.2.2. As a control, at the end of each experiment, an E or N-cadherin specific antibody was added to the cell culture medium and the measurements were re-recorded.

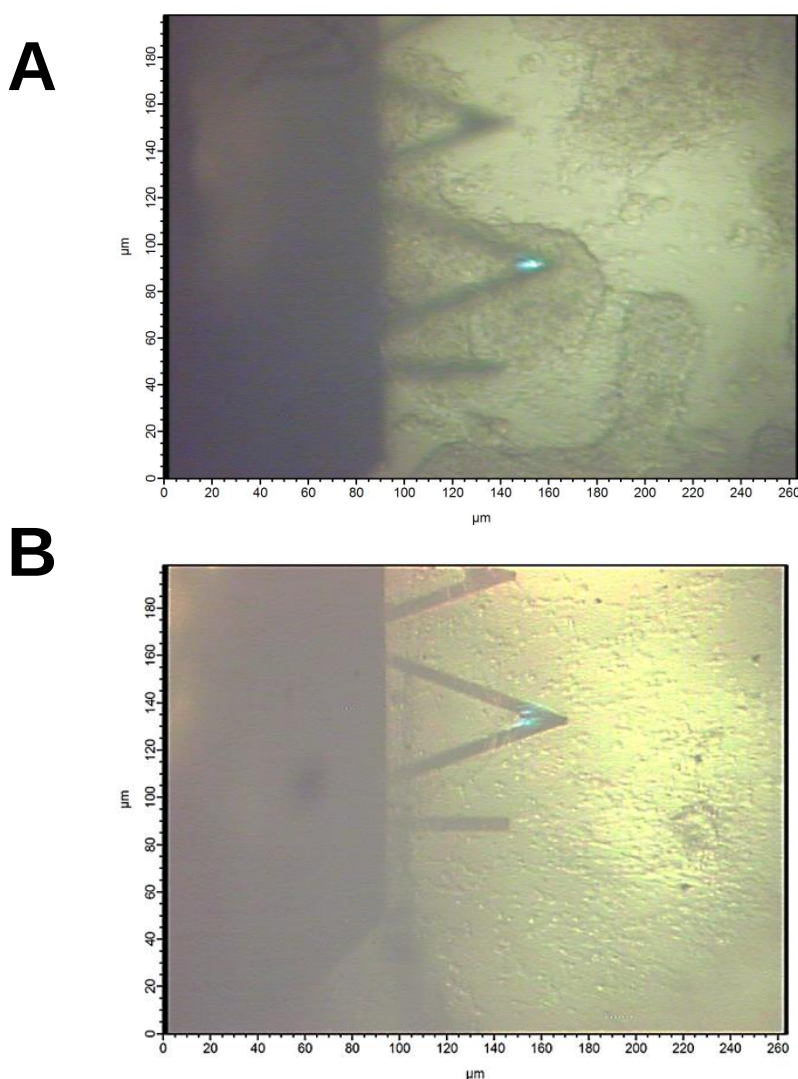


**Figure 5.2. Phase contrast image of a functionalized AFM tip located over different cell samples. (A) mES cells (B) NP cells.**

### **5.2.2. AFM tip functionality test**

Prior to rupture force measurements on cells, AFM tips functionalized with E- or N-cadherin were first tested for their ability to form specific bonds with

surfaces functionalized with the same type of cadherin, to verify their functionality. E- or N-cadherin ectodomains were attached to AFM tips according to the procedure described in Chapter III, Section 3.2.2.1. and brought into and out of contact with a functionalized silicon surface according to the procedure described in Chapter III, Section 3.2.2.2. The frequency and magnitude of specific adhesion events was then monitored. If the force was in a similar range to that recorded previously and the frequency was within the range of 10% to 20% the tip could be used for force measurements. Validated functionalized AFM tips were kept in HEPES buffer containing EGTA until recording force measurements on cells to prevent any damage to the cadherin ectodomains due to drying.

At the beginning of each experiment force measurements were first carried out in the buffer including EGTA. The frequency of cadherin adhesion events was found always to be low (<5%). Then functionalized tips and surfaces were washed at least three times in buffer and placed into HEPES buffer containing  $\text{Ca}^{2+}$  for at least 15 minutes. Force measurements were then carried out once again. If the frequency recovered and was within the range of 10% to 20% the tip was chosen for force measurements on cells.

Because these bond formations reoccurred once the cadherin construct was again in the presence of  $\text{Ca}^{2+}$  ions, presumably due to the ectodomains reassuming their original rod like confirmation (see Chapter III, Section 3.3.2.) which is more conducive to facilitate cadherin bond formations, it was assumed, that this AFM tip was suitable to be used for force measurements on cells.

## 5.3. Results and Discussion

### 5.3.1. E-Cadherin CFS on mES cells

To confirm the functionality of the tips employed in this study, before force measurements on cells were taken, AFM tips were tested for their ability to produce specific adhesion events on surfaces functionalized with the same type of cadherin as on the tip (as described in Section 5.2.2.). Figure 5.3. shows example data for this type of functionality test. AFM tips were kept in EGTA prior to measurements and the first measurement was made in HEPES buffer containing EGTA (Fig.5.3.A) to probe cadherin interactions when in their inactivated state (See Chapter III, Section 3.3.2.). Subsequently a second measurement was made in buffer containing  $\text{Ca}^{2+}$  ions to measure cadherin interactions when the ectodomains are in their activated state (Fig.5.3.B). Figure 5.3.C shows the frequency analysis data for this type of tip validation. For the data shown in the presence of EGTA, less than 4% of all force curves showed specific adhesion whereas in the presence of  $\text{Ca}^{2+}$  more than 19% of all force curves showed specific adhesion. This type of binding behaviour was consistent with data obtained in Chapter III, Section 3.3.2. Although the frequency of specific adhesion was lower in the presence of EGTA the magnitude of the modal rupture force of cadherin bonds varied only little in these measurements compared to  $\text{Ca}^{2+}$  treated samples (EGTA, 31 pN;  $\text{Ca}^{2+}$ , 44 pN). This was important as the measured forces were in the same range as those observed in Chapter III and the obtained forces in EGTA and  $\text{Ca}^{2+}$  were similar which suggests that the same interaction was observed although the binding frequency declined in EGTA.

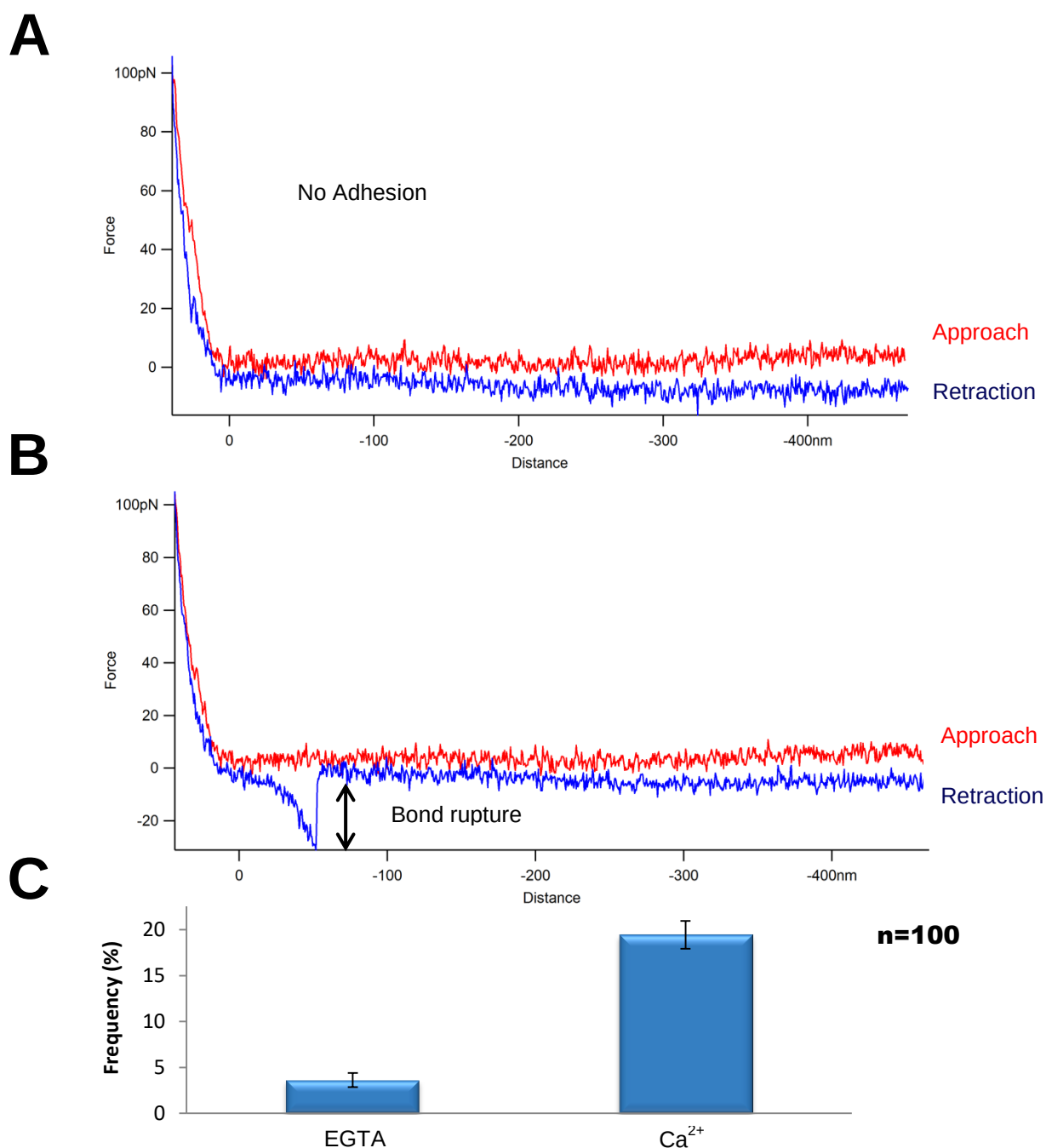


**Figure 5.3.** AFM force measurement curves recorded during a tip functionality test. Force measurements were first carried out in EGTA and then in  $\text{Ca}^{2+}$  containing HEPES buffer. (A) In the presence of EGTA specific adhesion events were largely absent from the force distance curves. (B) In the presence of  $\text{Ca}^{2+}$  ions, specific adhesion events could be observed in the FD curves. (C) Data shown represent the frequency of specific bond rupture force values measured in 100 force measurements using the same AFM tip. In the presence of EGTA only 3.6% of all force curves showed specific adhesion. In the presence of  $\text{Ca}^{2+}$  ions, specific adhesion increased and 19.4% of all force curves showed specific adhesion. Error bars shown represent the confidence interval based on the margin of error.

Tips confirmed to have functionality were then employed for experiments on mES cells. In these experiments, force-distance (FD) curves showing specific cadherin unbinding events were defined as those curves with non-linear profiles prior to the adhesion jump-off event, and occurring within the first 300 nm away from the cell surface (see Section 5.2.1. above). Figure 5.4.A shows the typical appearance of a specific adhesion event recorded on the surface of a mES cell with a tip speed of  $1 \mu\text{m s}^{-1}$ . Curves showing unspecific adhesion between the tip and the cell surface were identified by force jumps that occurred at 0 nm (Fig.5.4.B) or further than 300 nm from the cell surface or had a linear appearance prior to bond rupture (see Chapter III, Section 3.3.1.). Unspecific binding curves were attributed to interactions between the underlying tip and the cell membrane and were eliminated from subsequent analysis, as their origin could not easily be ascribed considering various factors such as the molecular dimensions of the cadherin ectodomains, the flexibility of the cell membrane and the composition of the cell culture medium.

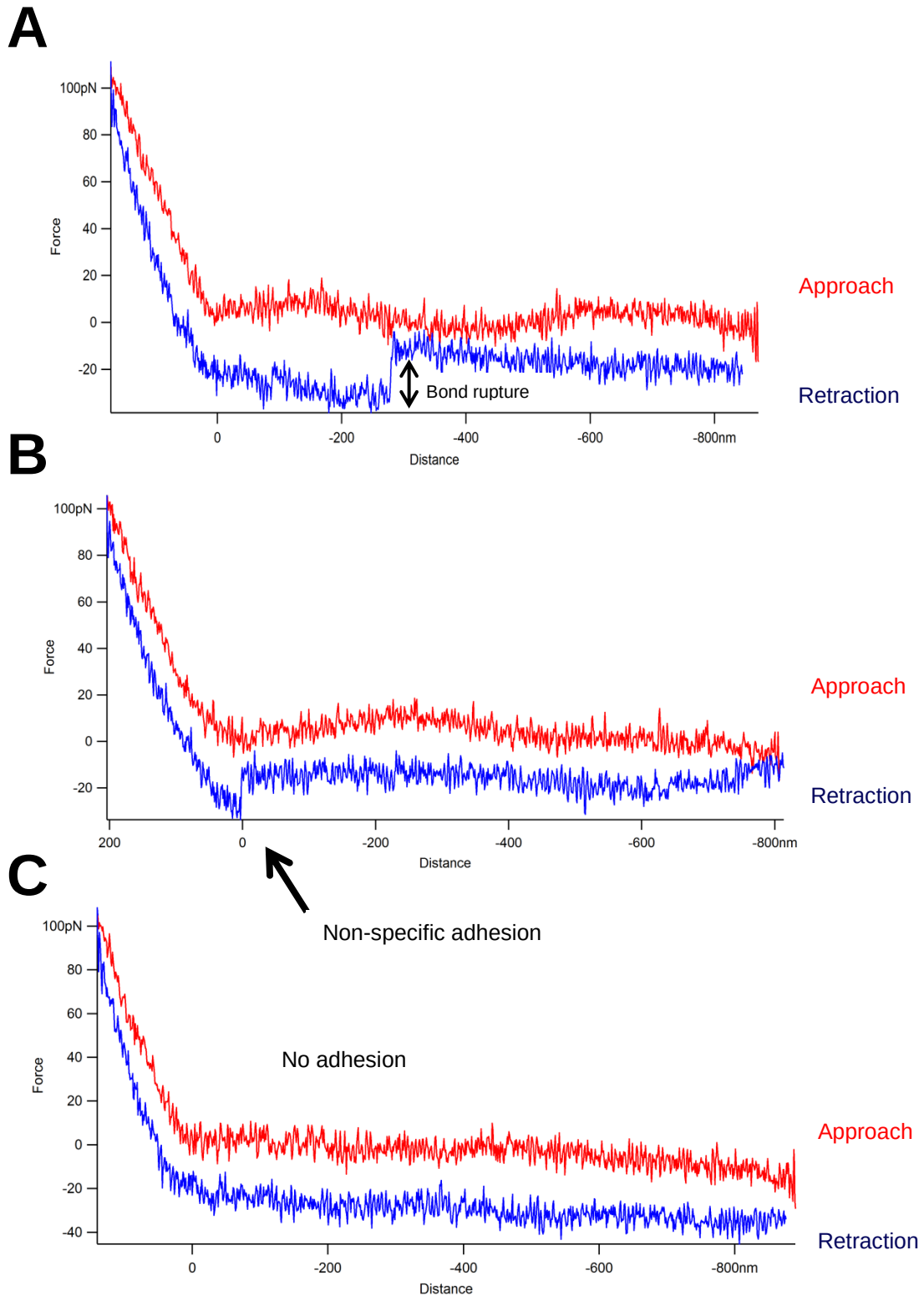


**Figure 5.4. AFM force-distance (FD) curves recorded between AFM tips functionalized with E-cadherin and mES cells. As in SMFS measurements the red line represents the approach and the blue line the retraction of the AFM tip. (A) Typical appearance of an unbinding curve showing specific adhesion. The extension curve shows nonlinear stretching behaviour preceding bond rupture and occurs less than 300 nm away from the cell surface. (B) Example force curve for unspecific binding. The bond rupture occurs directly (at 0 nm distance) on the cell membrane. (C) Example force curve obtained from interactions of a blank AFM tip with a mES cell surface. No binding events could be observed in this type of measurement.**

Figure 5.4.C. shows the AFM curve appearance obtained after measuring the adhesion force of a blank AFM tip and the surface of a mES cell. It can be seen in that the distinct force jump found in the force curves of Fig.5.4.A and 5.4.B, is absent in the force curve shown. This result thus served as a negative control and provided more evidence for the authenticity of the interactions being measured being indeed due to cadherin interactions.

The data obtained on mES cells were analysed according to the procedure described in Chapter III, Section 3.2.2.2. Similar to SMFS experiments, only about 10%-20% of all FD curves recorded showed specific adhesion (Fig.5.4.A). The rest of the force-distance curves showed unspecific adhesion (Fig.5.4.B) or no adhesion events at all (Fig 5.4.C). Figure 5.5. shows a typical distribution of specific E-cadherin bond rupture force values that were recorded on the surface of mES cells, at a tip velocity of  $1 \mu\text{m s}^{-1}$ . As a further control, at the end of each experiment, antibodies specific for either E- or N-cadherin were pipetted into the cell culture medium in which the force measurement was carried out (see Section 5.3.2. below) and force measurements re-recorded.

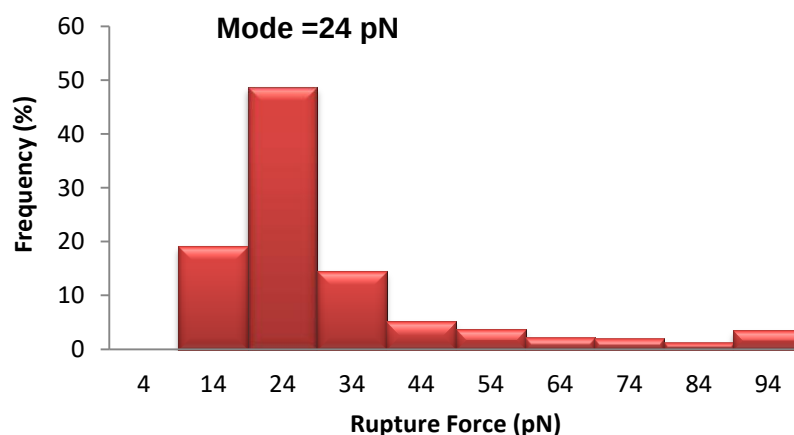


Figure 5.5. Example distribution of 1500 bond rupture force values. The specific bond rupture forces shown were measured at a tip speed of  $1 \mu\text{m s}^{-1}$  and were fitted using the in-house developed program Fdist<sup>63, 109</sup> to determine the mode force of the distribution (see Chapter II, Section 2.5.). The data was then plotted as a histogram with a bin size of 10 pN to show the distribution of the specific rupture force magnitudes centred around the mode force (24 pN).

### **5.3.2. Negative control: Antibody block of cadherin interactions on mES cells**

In order to show that the observed specific adhesion events were indeed due to rupture of cadherin bonds, force measurement experiments were carried out in the presence of either an E or N-cadherin specific antibody using the same AFM tip (Fig.5.6.). For interactions of E-cadherin coated tips with mES cell surfaces, the addition of an E-cadherin specific antibody resulted in a significant decline in specific cadherin bond formation. For the experiment shown in Figure 5.6.A (recorded with one tip) the frequency of specific cadherin adhesion events for example decreased in response to the antibody treatment from 11% to 2%.

Interestingly, for the same tip the addition of an antibody specific for the N-cadherin (i.e. the cadherin type not functionalized to the AFM tip), however, did not reduce cadherin bond formation and instead slightly increased the frequency of specific adhesion events to 18%. This may be attributed to the N-cadherin specific antibody binding to N-cadherin on the surface of mES cells and increasing the accessibility of E-cadherin ectodomains on the AFM tip to native E-cadherin molecules on the cell surface.

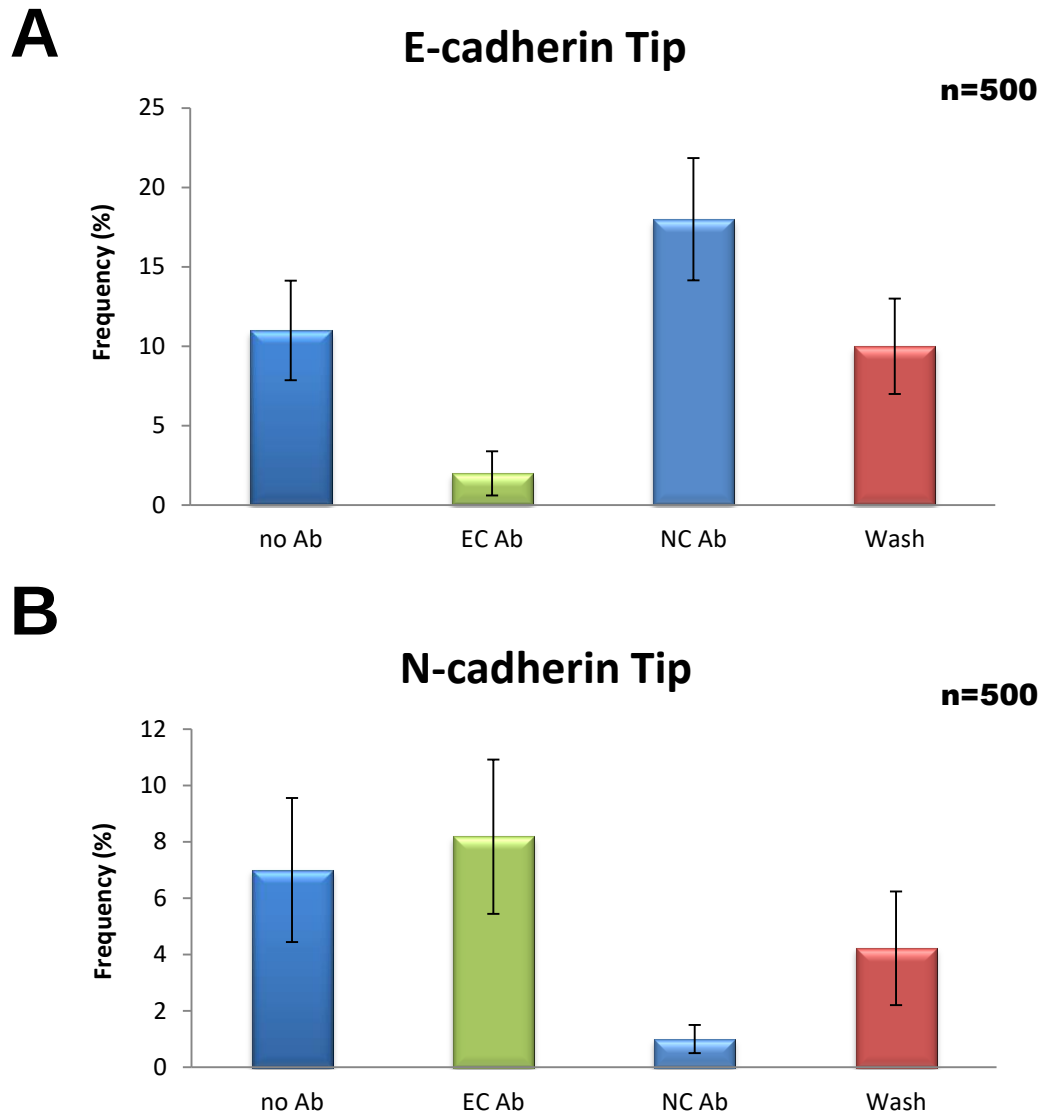


**Figure 5.6.** Example data for cadherin blocking experiments. The same AFM tip was used to measure the tip-surface interaction 500 times, at different positions for each treatment, at  $1 \mu\text{m s}^{-1}$  over a distance of 500 nm. Data shown represent the specific rupture force values measured in the experiments. (A) An E-cadherin tip is exposed to antibodies specific for E-cadherin (EC) and N-cadherin (NC) during a force measurement on mES cells. The antibodies (Ab) were subsequently removed by washing the cell sample and AFM tip with cell culture medium (Wash). After washing the AFM tip and sample surface in cell culture medium the effects of the antibody were in part reversed and higher frequencies of specific E-or N-cadherin bonds could be observed again. (B) An N-cadherin tip during a force measurement on mES cells is exposed to antibodies specific for E-cadherin and N-cadherin. The experimental procedure was kept the same as for E-cadherin. In both experiments the relative frequency of specific cadherin bond formation was greatly reduced in the presence of the antibody specific for the cadherin type on the AFM tip. Addition of an antibody not specific for the cadherin type functionalized to the AFM tip showed no inhibition of the homophillic cadherin adhesion. The increase of specific adhesion seen for the E- or N-cadherin tip in response to the antibody specific for the other type of cadherin could have been caused by a decline in unspecific adhesion of the cadherin ectodomain on the AFM tip (see main text, Section 5.3.2.). Error bars shown represent the confidence interval based on the margin of error.

After washing the cell sample for at least three times with cell culture medium, cadherin mediated adhesion events returned to frequencies close to their initial frequencies (10%). Similarly, when force measurements on mES cells were carried out with an N-cadherin coated AFM tip and exposed to an N-cadherin specific antibody, the frequency of specific binding events decreased from 7% to 1% (Fig.5.6.B). After washing the cell sample with cell culture medium the frequency of specific adhesion increased but remained at a lower frequency than the original sample (4.22%). For the same tip, addition of an antibody specific for E-cadherin did not reduce the relative frequency of specific adhesion events, but again lead to a small increase in specific adhesion (8.18%), again possibly due to an increased accessibility of the N-cadherin molecules on the cell surface.

These results demonstrated that it was possible to record the formation of molecular bonds between recombinant cadherin constructs on the AFM tip and cadherin on the surface of mES cells. These bonds could be blocked by specific antibodies but not by unspecific antibodies. This provided evidence for the specificity of the molecular interactions measured in AFM experiments on mES cells.

In addition it should be noted that by adding an antibody specific for e.g. E-cadherin the frequency of specific adhesion events recorded with an E-cadherin coated tip decreased but did not disappear altogether. When specific homophillic adhesion was blocked by the specific antibody it is therefore conceivable that the homophillic E-cadherin bonds were not entirely blocked by the antibody or that the E-cadherin construct was binding to another type of cadherin, such as N-cadherin on the cell surface of mES cells or possibly to

other types of cell adhesion molecules (CAM's) such as integrins <sup>166</sup> or other surface proteins <sup>167</sup>. The remaining adhesion that can be observed in interactions blocked with specific antibodies could, thus, be attributed to remaining homophillic, heterophillic or heterotypic binding of the cadherin ectodomains bound to the AFM tip.

### **5.3.3. Effect of tip speed on cadherin interactions on mES cells**

Before measurements on cells were carried out, E- cadherin coated AFM tips were tested for their ability to produce specific adhesion events by applying the tip functionality test procedure detailed in Section 5.2.2. above. Tips validated in this way were then selected for force measurements. E-cadherin bond rupture forces were measured at four different AFM tip speeds ( $1 \mu\text{m s}^{-1}$ ,  $750 \text{ nm s}^{-1}$ ,  $500 \text{ nms}^{-1}$ ,  $250 \text{ nm s}^{-1}$ ). The procedure described in Chapter III, Section 3.2.2.2. was then used to analyse the results. Tip-surface interactions were recorded 500 times at three different positions over the cell surface for each AFM tip (vertical distance  $1 \mu\text{m}$ ).

Figure 5.7. shows example distributions of rupture forces recorded for each of the tested tip speeds. As can be seen in Figure 5.7. and 5.8.A, the modal rupture force required to rupture cadherin bonds decreased in as the tip speeds were increased. The data shown represent three repetitions of the same experiment recorded, using three different AFM tips. The rupture forces had their highest values at  $250 \text{ nm s}^{-1}$ , where a modal force of  $45 \text{ pN} \pm 1.4 \text{ pN}$  was required to rupture formed cadherin bonds. The standard error of the mode was estimated using the procedure described in Chapter II, Section 2.5.

As the tip speed was increased up to a maximum of  $1 \mu\text{m s}^{-1}$  the modal rupture forces decreased to values as low as  $23 \text{ pN} \pm 0.4 \text{ pN}$ . Figure 5.8.B shows the frequency analysis of cadherin bond rupture events. Specific adhesion events occurred at similar low probabilities (8% to 16%) relative to all force measurements taken, regardless of changing tip speeds which, suggested that the rupture forces observed were predominantly due to rupture of single molecule bonds according to Poisson statistics<sup>86, 86</sup>.

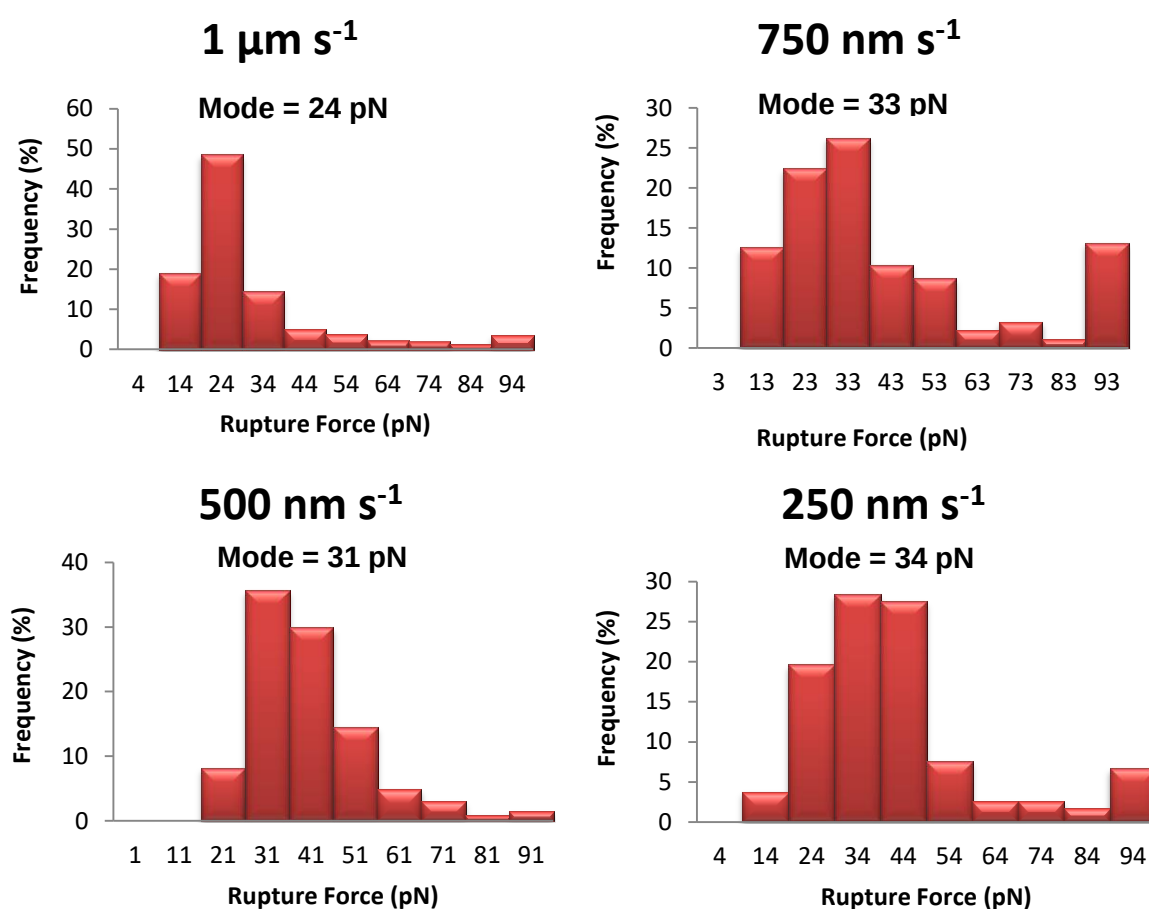


**Figure 5.7. Rupture force distributions of E-cadherin bonds formed on the surface of mES cells.** Tip-surface interactions were recorded 500 times at three different positions over a distance of  $1 \mu\text{m}$  from the cell surface for each AFM tip. The data shown represent 3 repetitions of the same experiment recorded using 3 different AFM tips. As the tip speed was decreased modal bond rupture forces between E-cadherin molecules increased accordingly. ( $1 \mu\text{m s}^{-1}$ , 24 pN;  $750 \text{ nm s}^{-1}$ , 33 pN;  $500 \text{ nm s}^{-1}$ , 31 pN;  $250 \text{ nm s}^{-1}$ , 34 pN). The force values were fitted using the in-house developed program Fdist<sup>63, 109</sup> to determine the mode force of the distribution (see Chapter II, Section 2.5.). The data was then plotted as a histogram with a bin size of 10 pN to show the distribution of the specific rupture force magnitudes centred around the mode force.

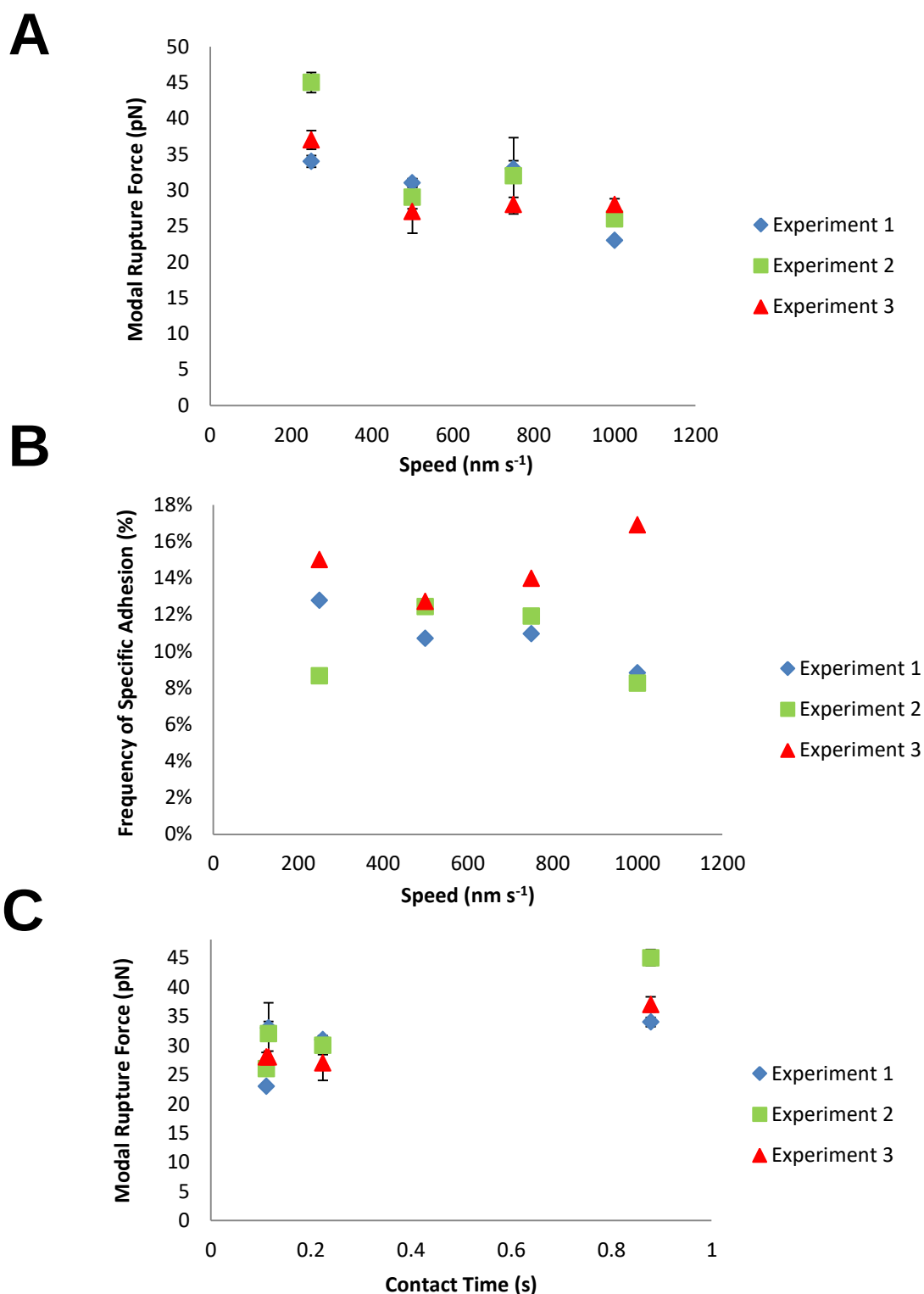


**Figure 5.8.** Rupture forces of E-cadherin bonds on mES cells as a function of AFM measurement speed. Data shown represent three repetitions of the same experiment using three different AFM tips. Force measurements were carried out at four different tip speeds that were adjusted to the cell based experiments ( $1 \mu\text{m s}^{-1}$ ,  $750 \text{ nm s}^{-1}$ ,  $500 \text{ nm s}^{-1}$ ,  $250 \text{ nm s}^{-1}$ ). Tip-surface interactions were measured and recorded 500 times at three different positions over a distance of  $1 \mu\text{m}$  from the cell surface. (A) As the tip speed was increased E-cadherin bond rupture forces decreased in a negative linear fashion. (B) The frequency at which specific binding between E-cadherin receptors occurred showed no significant change in response to changing tip speeds. (C) Dependence of specific bond rupture force on estimated contact time. E-cadherin modal bond rupture forces increased as the contact time increased. Error bars shown represent the standard deviation of the modal value of recorded forces.

The behaviour of the rupture forces of E-cadherin bonds measured at different tip speeds on the cell surface of mES cells also bore strong resemblance to the SMFS results presented in Chapter III, Section 3.3.3. In SMFS measurements of cadherin interactions bond rupture forces also declined with an increase in tip speed, at similar force magnitudes at the respective tip speeds (e.g. in CFS experiments:  $1 \mu\text{m s}^{-1}$ ,  $23 \text{ pN} \pm 0.4 \text{ pN}$  ; in SMFS experiments:  $26 \text{ pN} \pm 1 \text{ pN}$  etc.). This provided further evidence that the molecular interactions being measured on the surface of mES cells were in fact due to cadherin bonds being ruptured, since the response of the molecular bonds under investigation here, responded to external applied force in a similar manner as the observed bonds in SMFS experiments in Chapter III.

To understand the origin of the decreasing force, the effect of probe-sample contact time was again considered. Due to the soft nature of cell surfaces and the considerable error in defining the exact point of probe-cell contact (e.g  $z=0$ ) it was however very difficult to precisely measure contact time.<sup>168</sup>. Hence the contact time of the tip with the cell surface could only be estimated using the same approach as for SMFS experiments (as detailed in Chapter III, Section 3.3.4.). The total estimated time of contact time of the AFM tip with the cell surface decreased as the tip speeds were increased.

Figure 5.8.C. shows the modal rupture forces plotted against the estimated tip-surface contact time. The modal rupture force for E-cadherin can be seen to increase directly with increasing tip-surface contact time. The highest modal rupture force (45 pN) occurred at 0.8 seconds estimated contact time,

whereas the lowest modal rupture force (23 pN) was measured at 0.11 seconds estimated contact time.

As was observed during the SMFS measurements in Chapter III, Section 3.3.3., the results obtained differ from other studies in that the force required to rupture cadherin bonds decreased with increasing tip speeds. In DFS, when proteins are brought into contact with one another, the rupture force of their formed bond usually increases linearly as a function of the natural logarithm of the loading rate <sup>104</sup>. The reason that the obtained results differ from those studies could be attributed to the fact that the tip dwell time was kept at 0 seconds which means that the contact time varied according to the tip speed used e.g. slower tip speeds resulted in longer contact times. With the increasing contact time more cadherin subunits (EC's) would thus be able to bind and potentially form stronger bonds. This binding behaviour seen here for cadherin bonds on undifferentiated mES cells was however consistent with that observed in Chapter III, and thus it was now possible to also study N-cadherin interactions on undifferentiated mES cells with the aim of directly comparing the results to the SMFS results presented in Chapter III.

#### **5.3.4. N-Cadherin CFS on mES cells**

Before measurements on cells were carried out, N-cadherin coated AFM tips were tested for their ability to produce specific adhesion events by applying the tip functionality test procedure detailed in 5.2.2. above. Tips validated in this way were then selected for force measurements.

N-cadherin coated AFM tips were then brought into and out of contact with the cell surface of mES cells to at four different tip speeds as described for the

study of E-cadherin interactions on mES cells above (Section 5.3.3.). The data shown in Fig.5.9. and Fig.5.10 represents two repetitions of the same experiment recorded using two different AFM tips. Figure 5.9.shows the specific bond rupture force distribution at each of the tested tip speeds.

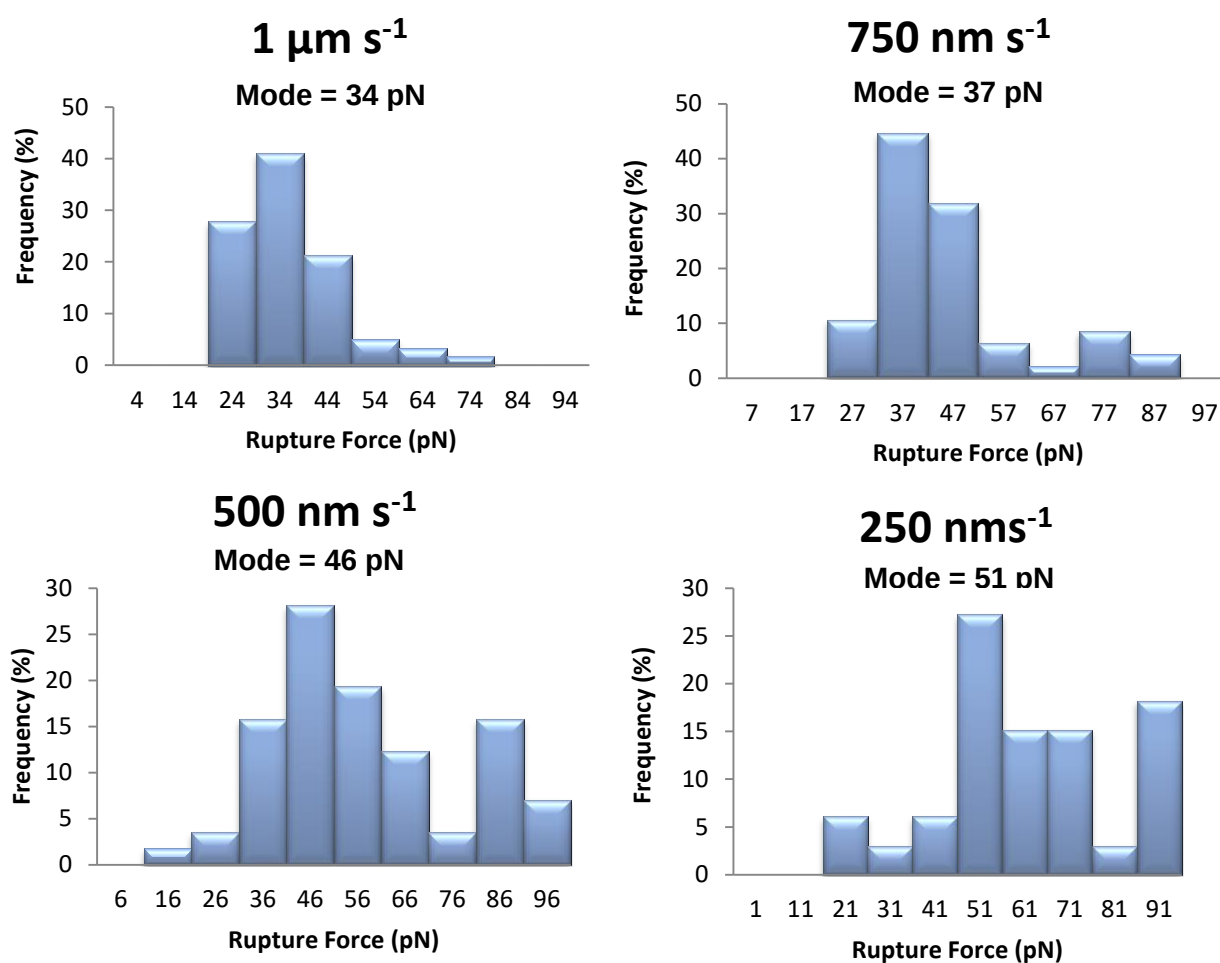


Figure 5.9. Histograms of N-cadherin bond rupture forces probed on the surface of mES cells and recorded for two AFM tips. Tip-surface interactions were recorded 500 times at three different positions over a distance of 1  $\mu\text{m}$  from the cell surface for each AFM tip. As the tip speed was reduced, higher modal rupture forces started to occur and the distribution of rupture forces shifted towards higher forces. The specific bond rupture forces were fitted using the in-house developed program Fdist<sup>63, 109</sup> to determine the mode force of the distribution (see Chapter II, Section 2.5.). The data was then plotted as a histogram with a bin size of 10 pN to show the distribution of the specific rupture force magnitudes centred around the mode force.

At the slowest of the tested tip speeds (250 nm  $\text{s}^{-1}$ ) the mode of the rupture force of N-cadherin bonds reached its highest value (58 pN  $\pm$  2.6 pN). This value was similar (i.e. <10 pN difference) to that recorded for E-cadherin

bonds at the same velocity (ca. 45 pN). As the tip speed was increased to 1  $\mu\text{m s}^{-1}$  the modes of N-cadherin rupture forces decreased consistently, reaching their lowest value at 1  $\mu\text{m s}^{-1}$  with a value of 30 pN  $\pm$  1.9 pN (Fig.5.10.A). This value for the modal rupture force was again similar to that measured for E-cadherin on mES cells at 1  $\mu\text{m s}^{-1}$  (23 pN  $\pm$  0.4 pN). The binding frequency of specific adhesion events remained largely unaffected by the change in tip speed and stayed between 5% and 18 %, with no discernable trends in response to changing tip speeds (Fig.5.10.B).

The rupture force modes increased with an increase in estimated contact time, as was also observed for E-cadherin interactions on mES cells. This similarity was not surprising given the fact that classical type I cadherins E- and N-cadherin have a high sequence similarity in their EC1-EC5 and EC1 subunits (48% / 58% respectively) <sup>130</sup>. It is interesting to note however that Panchoran *et al.* <sup>165</sup> reported quantitative differences in binding strength and reactive compliance for E- and N-cadherin bonds probed with AFM on the surface of chinese hamster ovary (CHO) cells (see Section 5.1.). Possibly the reason we could not observe the same discrepancy between E-and N-cadherin bonds on the surface of mES cells was that they each have distinct functions on mES cells.

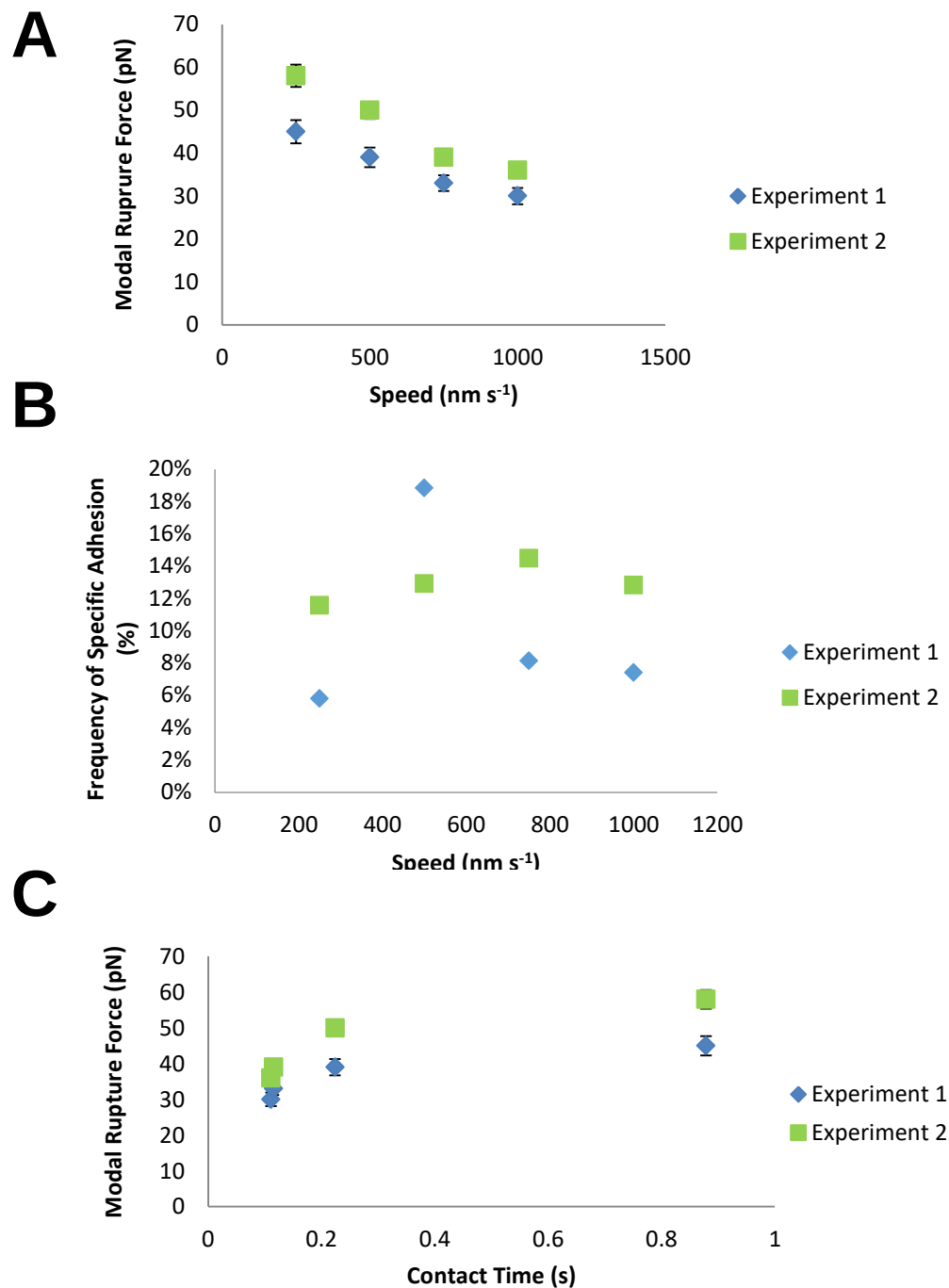


**Figure 5.10.** Rupture forces of N-cadherin bonds on mES cells in dependence on the AFM tip speed. Force measurements were carried out at four different tip speeds ( $1 \mu\text{m s}^{-1}$ ,  $750 \text{ nm s}^{-1}$ ,  $500 \text{ nm s}^{-1}$ ,  $250 \text{ nm s}^{-1}$ ). Tip-surface interactions were measured and recorded 500 times at three different positions over a distance of  $1 \mu\text{m}$  from the cell surface for two AFM tips. (A) Rupture forces of N-cadherin bonds decreased with increasing AFM tip speeds. (B) Relative binding frequencies of cadherin interactions were not affected by a change in tip speed and stayed between 5% and 19%. (C) Dependence of N-cadherin bond rupture force on estimated contact time. N-cadherin bond strength increased as the contact time was increased. Error bars indicate the standard deviation of the modal value of recorded forces.

E-cadherin has a high surface expression relative to N-cadherin as shown in our IHC staining experiments (see Chapter IV, Section 4.3.2.), therefore it is conceivable that E-cadherin functions as the main stress bearing unit in maintaining cell to cell adhesion between undifferentiated mES cells, whereas the proposed low bond resistance<sup>165</sup> of N-cadherin, which is needed for early developmental processes such as epithelial- mesenchymal transitions (EMT's)<sup>25</sup>, might not yet be required and thus N-cadherin remains in a pre-mature state on mES cells in the CFS experiments presented here.

The analysis of mES cells using IHC staining in Chapter IV, Section 4.3.2. revealed a lower relative expression level of N-cadherin compared to E-cadherin on the cell surface of mES cells. Interestingly, the lower expression levels of N-cadherin on the surface of mES cells did not correspond with lower rupture forces at any of the tested tip speeds. These results suggest that the expression level of N-cadherin on the cell surface did not affect the magnitude of the bond rupture forces recorded.

Furthermore, although heterophilic bonds of N-cadherin on the AFM tip with E-cadherin on the cell surface are conceivable based on our results from SMFS presented in Chapter III (Section 3.3.6.), the negative control using an N-cadherin specific antibody to block the interaction of N-cadherins (see Section 5.3.2.) could demonstrate the specific nature of N-cadherin adhesion measured on mES cells. This suggests that N-cadherin-E-cadherin bonds are unlikely to have occurred in these force measurements despite their potential for heterophilic or heterotypic adhesion.

### **5.3.5. Summary I: Combining SMFS and CFS on mES cells**

In summary it was possible to use the AFM based methodology developed in Chapter III, to study cadherin interactions on the surface of undifferentiated mES cells. Strong similarities could be observed when measuring the bond rupture forces of E- and N-cadherin, at different tip speeds on undifferentiated mES cells and on functionalized surfaces. Figure 5.11. shows the obtained results of SMFS and CFS on mES cells in the same graph.

E-and N-cadherin modal bond rupture forces decreased with increasing tip speeds and could be blocked by an antibody specific for the cadherin type on the AFM tip. This could provide evidence for the authenticity of the molecular bonds under investigation on the surface of mES cells and suggested that they were indeed due to cadherin interactions.

With a measurement technique that could yield single bond formations which could be blocked by cadherin specific antibodies and was transferrable to other cell types, it was now possible to study E- and N-cadherin interactions on mES cells as they differentiated into defined cellular lineages. In order to investigate if and how cadherin interactions would change as mES cells differentiated, the neuronal differentiation pathway was chosen to study these phenomena. For this purpose the procedure developed in Chapter IV (Section 4.2.2.) was utilized for deriving neuronal precursor (NP) cells from undifferentiated mES cells. The interactions of E and N-cadherin on these NP cells were then investigated using the same protocol for CFS as employed on mES cells.

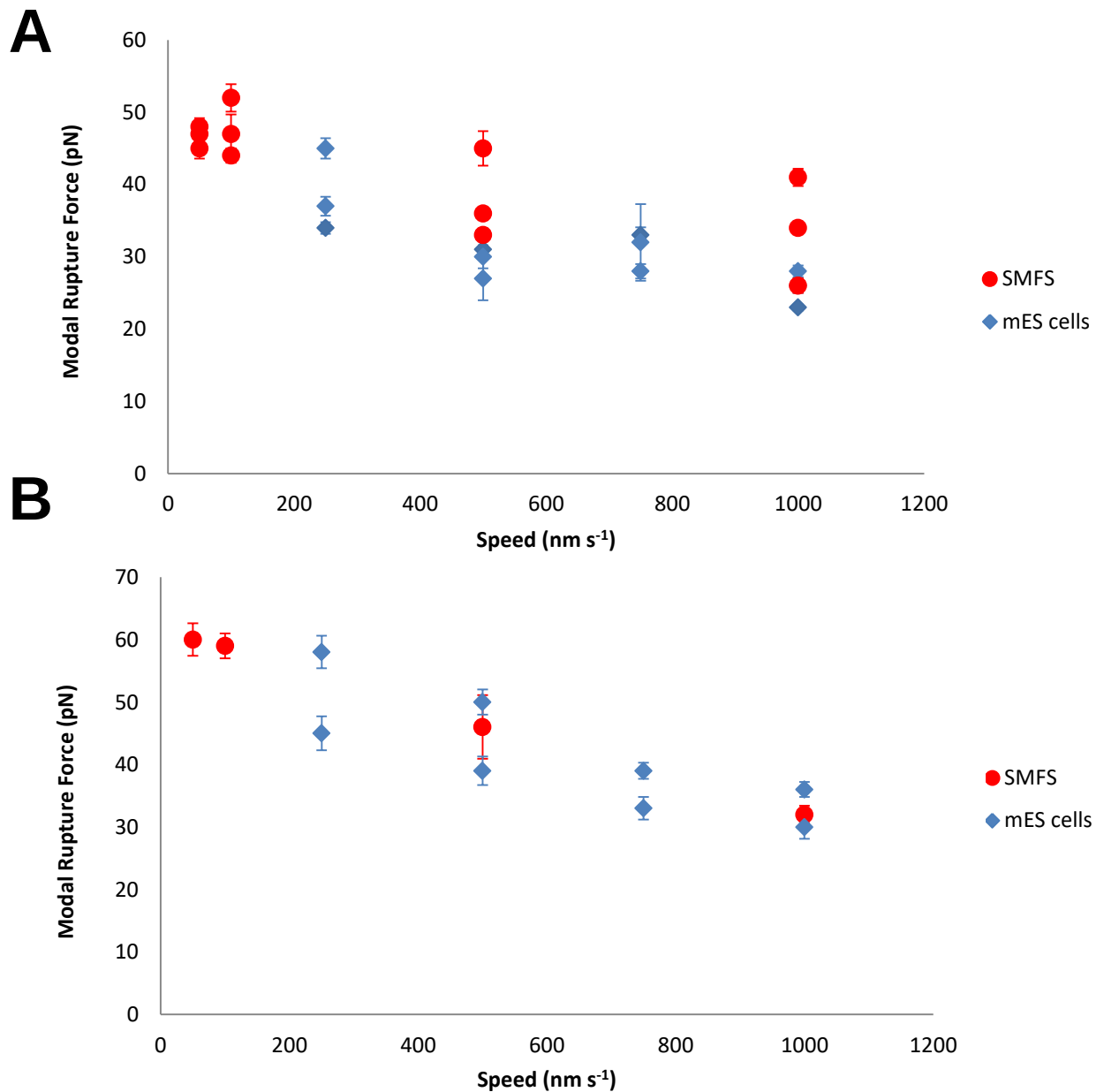


Figure 5.11. Comparison of results obtained for E- and N-cadherin interactions in SMFS and CFS on mES cells. (A) Comparison of bond rupture forces for E-cadherin. (B) Comparison of bond rupture forces for N-cadherin. Error bars shown represent the standard deviation of the modal value of recorded forces.

### 5.3.6. E-Cadherin CFS on NP cells

mES cells were differentiated into NP cells according to the procedure detailed in Chapter IV and prepared for analysis with the AFM. First, in order to demonstrate the functionality of the E-cadherin ectodomains on the AFM

tips, they were tested according to the procedure detailed in Section 5.2.2. above. Because measurements with the tips employed in these experiments produced similar E-cadherin bond rupture forces and similar binding frequencies, it was assumed that they were able to measure cadherin interactions on the surface of NP cells in a similar manner as previously measured on mES cells and functionalized surfaces. The results for this initial tip functionality test are shown in Figure 5.12. AFM tips validated in this way were then used for NP cell force measurements.

The force curves in Figure 5.13. show examples of curves recorded on NP cells. The same criteria that were used in force measurements on mES cells to distinguish between specific, non-specific and no adhesion were also used in force measurements on NP cells (as defined in Section 5.3.1.). Figure 5.13.A shows the appearance of a force curve recorded on cells showing specific adhesion; Figure 5.13.B and Figure 5.13.C show unspecific binding and the absence of binding events during a force measurement respectively.

E-cadherin coated AFM tips were brought into and out of contact with the cell surface of NP cells to at four different tip speeds as described for the study of E-cadherin interactions on mES cells (Section 5.3.3.). Unfortunately due to time constraints this experiment could only be carried out for one AFM tip. However, due to the number of measurements recorded and the tip test data it was possible to compare the results to the data obtained in measurements on mES cells and functionalized surfaces.

Figure 5.14 shows the distribution of rupture forces at each of the tested tip speeds. The force required to rupture E-cadherin bonds was again found to decrease as the tip speed was increased. As can be seen in Figure 5.15.A the specific bond rupture forces had their highest value at  $250 \text{ nm s}^{-1}$ . Here, forces with a mode of  $78 \text{ pN} \pm 3.3 \text{ pN}$  were required to rupture formed cadherin bonds.

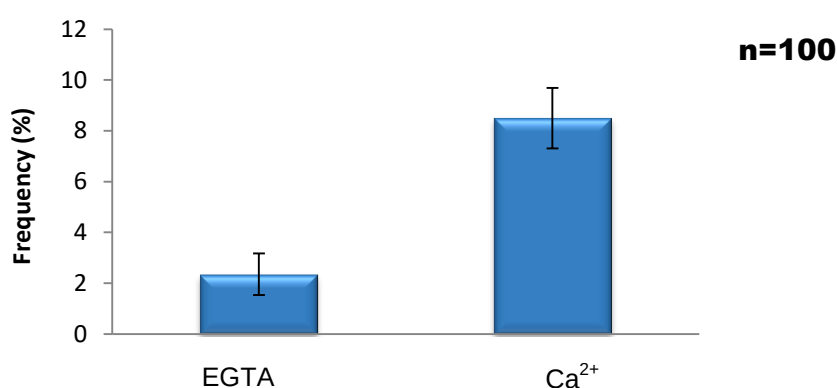


Figure 5.12. E-cadherin tip functionality test. Data shown represent the frequency of specific rupture force values measured in 100 force measurements using the same AFM tip. The frequency of specific adhesion events was reduced in the presence of EGTA. This demonstrated the functionality of the E-cadherin ectodomain the AFM tip prior to force measurements on NP cells. Error bars shown represent the confidence interval based on the margin of error.

As the tip speed was increased to a maximum of  $1 \mu\text{m s}^{-1}$ , the E-cadherin bond rupture forces decreased to values as low as  $50 \text{ pN} \pm 4.5 \text{ pN}$ . Specific cadherin adhesion events occurred with low probabilities (8% to 27%) regardless of changing tip speeds (Fig.5.15.B). However, it is noteworthy that the frequency of specific adhesion events for E-cadherin on NP cells at tip speeds of  $750 \text{ nm s}^{-1}$  and  $500 \text{ nm s}^{-1}$  was high compared to other measurements taken. According to Poisson statistics as long as the relative frequency of specific adhesion events is less than 30 %, >80% of the rupture events observed are due to single bonds<sup>85, 86</sup>. The results for binding frequency for tip speeds  $750 \text{ nm s}^{-1}$  and  $500 \text{ nm s}^{-1}$  lay just barely within this threshold (27%).

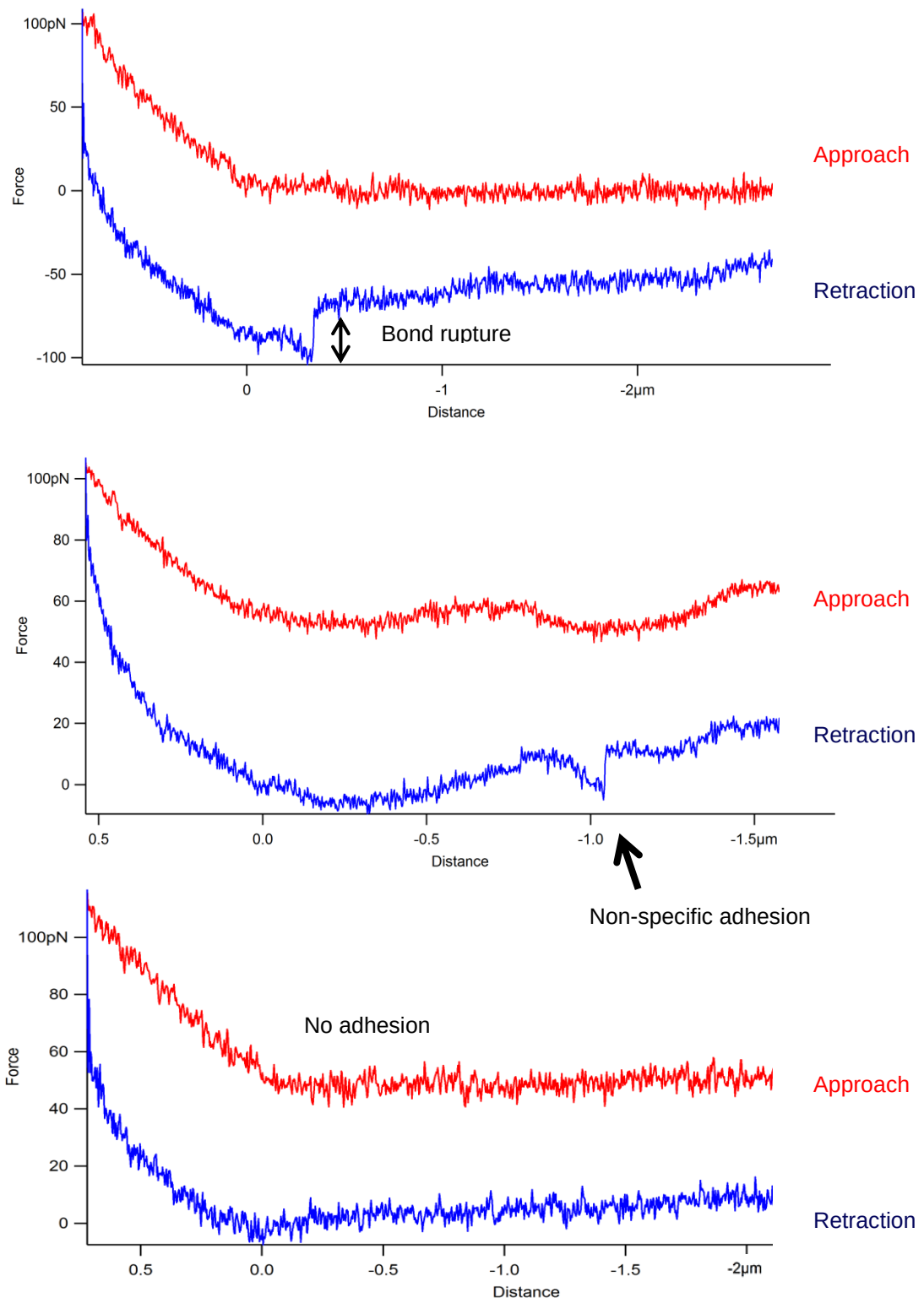


**Figure 5.13.** Force curves obtained in measurements with an AFM tip functionalized with E-cadherin on NP cells. (A) Typical appearance of an unbinding curve showing specific adhesion. The extension curve showed nonlinear stretching behaviour preceding bond rupture and occurred less than 300 nm away from the cell surface. (B) Example force curve for unspecific binding events. The rupture of a formed bond occurred more than 1 μm away from the cell surface and thus was unlikely to have been caused by cadherin interactions. (C) Example force curve obtained from interactions of a blank AFM tip with a NP cell surface. No binding events could be observed in these measurements.

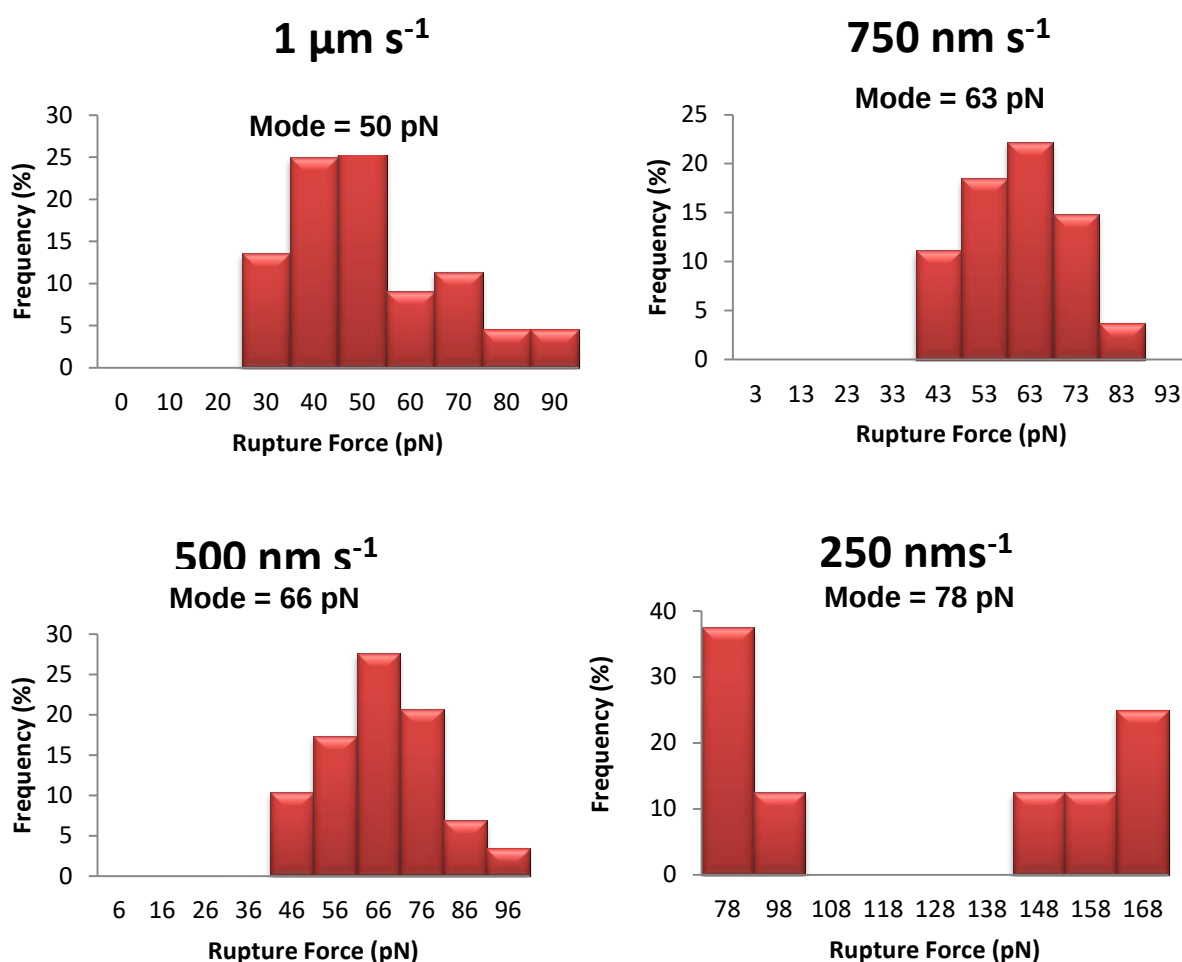


**Figure 5.14.** Statistical analysis of the specific bond rupture forces measured between an E-cadherin functionalized AFM tip and the surface of NP cells at tip speeds of  $1 \mu\text{m s}^{-1}$ ,  $750 \text{ nm s}^{-1}$ ,  $500 \text{ nm s}^{-1}$  and  $250 \text{ nm s}^{-1}$ . As the tip speed was decreased, rupture forces of E-cadherin bonds increased. This cannot be said with full confidence for data at  $250 \text{ nm s}^{-1}$  due to limited amounts of data available. The bond rupture forces were fitted using the in-house developed program Fdist<sup>63, 109</sup> to determine the mode force of the distribution (see Chapter II, Section 2.5.). The data was then plotted as a histogram with a bin size of 10 pN to show the distribution of the specific rupture force magnitudes centred around the mode force.

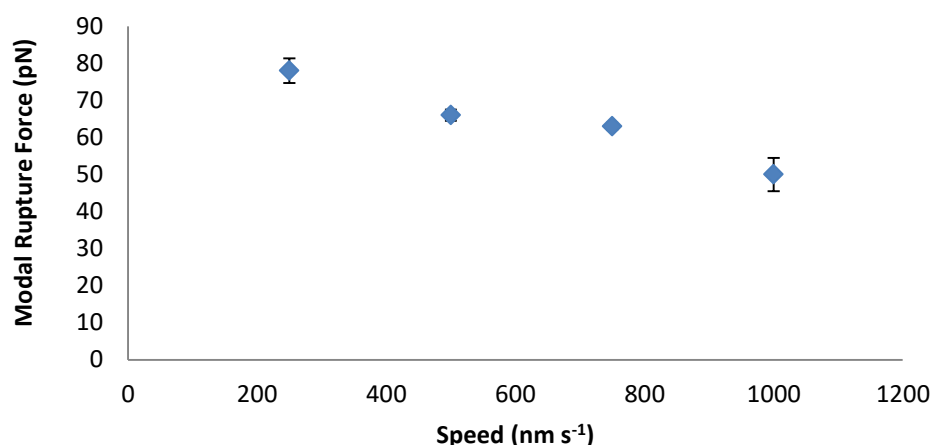
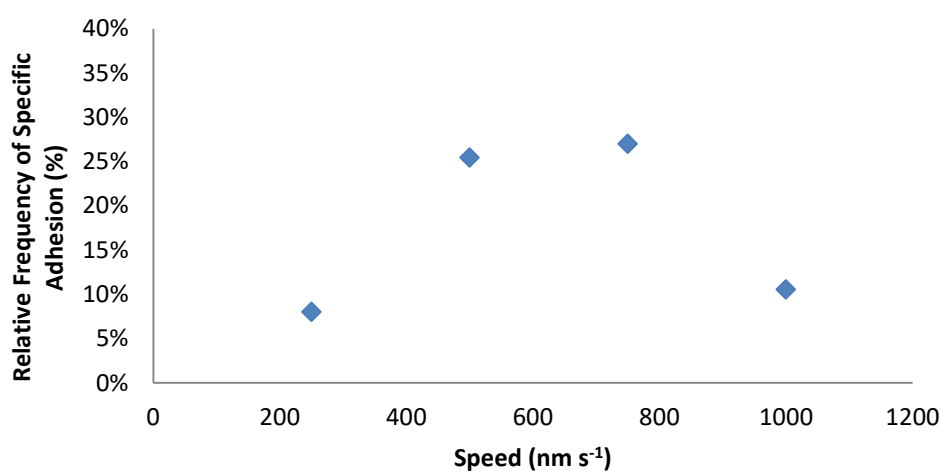
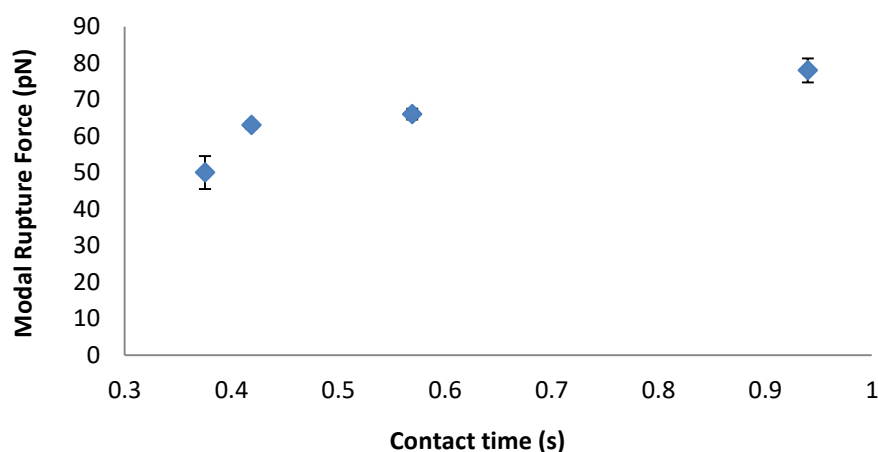
**A****B****C**

**Figure 5.15.** Rupture forces of E-cadherin bonds on NP cells as a function of the AFM tip speed. Force measurements were carried out at four different tip speeds. ( $1 \mu\text{ms}^{-1}$ ,  $750 \text{ nms}^{-1}$ ,  $500 \text{ nms}^{-1}$ ,  $250 \text{ nms}^{-1}$ ). Tip-surface interactions were measured and recorded 500 times at three different positions over a maximum distance of  $2 \mu\text{m}$  from the cell surface for one AFM tip. (A) As the tip speed was increased rupture forces between E-cadherin ectodomains decreased accordingly in a negative linear fashion. (B) The relative frequency at which specific E-cadherin binding occurred showed low variations in response to changing tip velocities and stayed between 8% and 27%. (C) E-cadherin mode bond rupture forces increased as the contact time increased. Error bars indicate the standard deviation of the modal value of recorded forces.

Overall, compared to mES cells and functionalized surfaces, the rupture forces of E-cadherin recorded on NP cells appeared to be higher for all tested tip speeds. Our analysis of NP cells using immunohistochemistry staining revealed a lower expression level of E-cadherin compared to N-cadherin on the surface of NP cells (see Chapter IV, Section 4.3.2.). The lower expression level of E-cadherin did not, however, correspond to lower rupture forces at the tested tip speeds here, but rather lead to higher mode rupture forces values in these measurements.

Figure 5.15.C. shows the modal rupture forces as a function of the tip-cell contact time. The highest modal rupture forces ( $78 \text{ pN} \pm 3.3 \text{ pN}$ ) occurred at 0.9 seconds contact time, whereas the lowest modal rupture forces ( $50 \text{ pN} \pm 4.5 \text{ pN}$ ) were observed at 0.37 seconds contact time. The data again suggests that a rise in tip-surface contact time lead to an increase in mode rupture force values.

The results obtained however must be observed with caution. First, given the high recorded relative frequency of specific binding events for tip speeds  $750 \text{ nm s}^{-1}$  and  $500 \text{ nm s}^{-1}$ , multiple cadherin bonds may have formed rather than single molecular bonds. Second, the increase in binding frequency could also be reflective of the limited amount of data available for this experiment, as due to time constraints the measurements could only be carried out for one AFM tip. However, the adhesion frequency (8%) and force mode values ( $40 \pm 1 \text{ pN}$ ) for the tip functionality test recorded at  $1 \text{ } \mu\text{m s}^{-1}$ , were consistent with that observed in the other SMFS experiments where single molecule bond measurements were made. The increase in force may therefore indeed reflect a change in the cadherin behaviour, rather than an increase in formed bonds.

It is also interesting to note that previously published results on (CHO) cells (as mentioned in Section 5.1.), also reported higher bond rupture forces for E-cadherin than for N-cadherin <sup>165</sup>. Although more experiments are clearly needed, the fact that higher forces of E-cadherin interactions were recorded in these studies only on NP and not for similar measurements on mES cells, suggests that changes may occur within the E-cadherin structure and/or linkage with intracellular structures as the mES cell turns into a NP cell e.g. so that it forms mechanically stronger bonds as the mES cell matures and differentiates along specific lineages.

### **5.3.7. N-Cadherin CFS on NP cells**

In order to demonstrate the functionality of the AFM tip used for force measurements on NP cells they were again first tested according to the procedure detailed in section 5.2.2. N-cadherin coated AFM tips were brought into and out of contact with the cell surface of NP cells at four different tip speeds. The experimental procedure was kept the same as for CFS of E-cadherin bonds on NP cells described above. Again due to time constraints this experiment could only be carried out for one AFM tip, however because measurements with the tip produced similar N-cadherin bond mode rupture forces at a tip speed of  $1 \mu\text{m s}^{-1}$  and had a similar binding frequency it was assumed that this tip was measuring the same cadherin interactions on the surface of NP cells as those previously measured on mES cells and functionalized surfaces. The results for this tip functionality test are shown in Figure 5.16.

Figure 5.17 shows the frequency distribution of rupture forces at each of the tested tip speeds. The force required to rupture N-cadherin bonds decreased as the tip speed was increased. Figure 5.17.A. shows the bond rupture forces as a function of the AFM tip speed. At the slowest of the tested tip speeds ( $250 \text{ nm s}^{-1}$ ) the mode of the rupture force of N-cadherin bonds reached a modal value of  $53 \text{ pN} \pm 24.1 \text{ pN}$  which was higher than the maximum modal rupture force required to disrupt E-cadherin bonds on mES cells at the same tip speed ( $45 \text{ pN} \pm 2.3 \text{ pN}$ ) (see Fig.5.8.). As the AFM tip speed was increased from  $250 \text{ nm s}^{-1}$  to  $750 \text{ nm s}^{-1}$  the modal rupture forces of N-cadherin on NP cells decreased from  $53 \text{ pN} \pm 24.1 \text{ pN}$  to  $35 \text{ pN} \pm 3.4 \text{ pN}$ . At the fastest of the tested tip speeds ( $1 \text{ } \mu\text{m s}^{-1}$ ) the modal rupture force increased to  $61 \text{ pN} \pm 2.1 \text{ pN}$  which represented an exception to other rupture force experiments, where rupture forces were found to decline with increasing tip speeds.

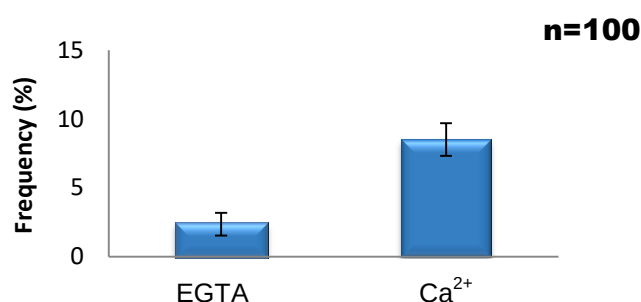


Figure 5.16. N-cadherin tip functionality test. Data shown represent the frequency of specific rupture force values measured in 100 force measurements using the same AFM tip. The frequency of specific adhesion events was reduced in the presence of EGTA. This demonstrated the functionality of the N-cadherin ectodomain the AFM tip prior to force measurements on NP cells. Error bars shown represent the confidence interval based on the margin of error.

The binding frequency of specific adhesion showed no clearly discernible trend in response to changing tip speeds and stayed between 9% and 20 % (Fig.5.18.B). This showed that the interactions were likely to have been dominated by single bond rupture events according to Poisson statistics<sup>85, 86</sup>.

Figure 5.18.C shows the dependence of bond rupture forces on estimated tip-cell contact time. The modal rupture force was found to increase with increasing contact time in a similar fashion as was observed for N-cadherin interactions on mES cells and functionalized surfaces, except for  $1 \mu\text{m s}^{-1}$  tip speed.

Our analysis of NP cells using IHC staining revealed a higher relative expression level of N-cadherin compared to E-cadherin on the surface of NP cells (see Chapter IV, Section 4.3.2.). The higher expression levels of N-cadherin did, however, not correspond to higher bond rupture forces at any of the tested tip speeds.

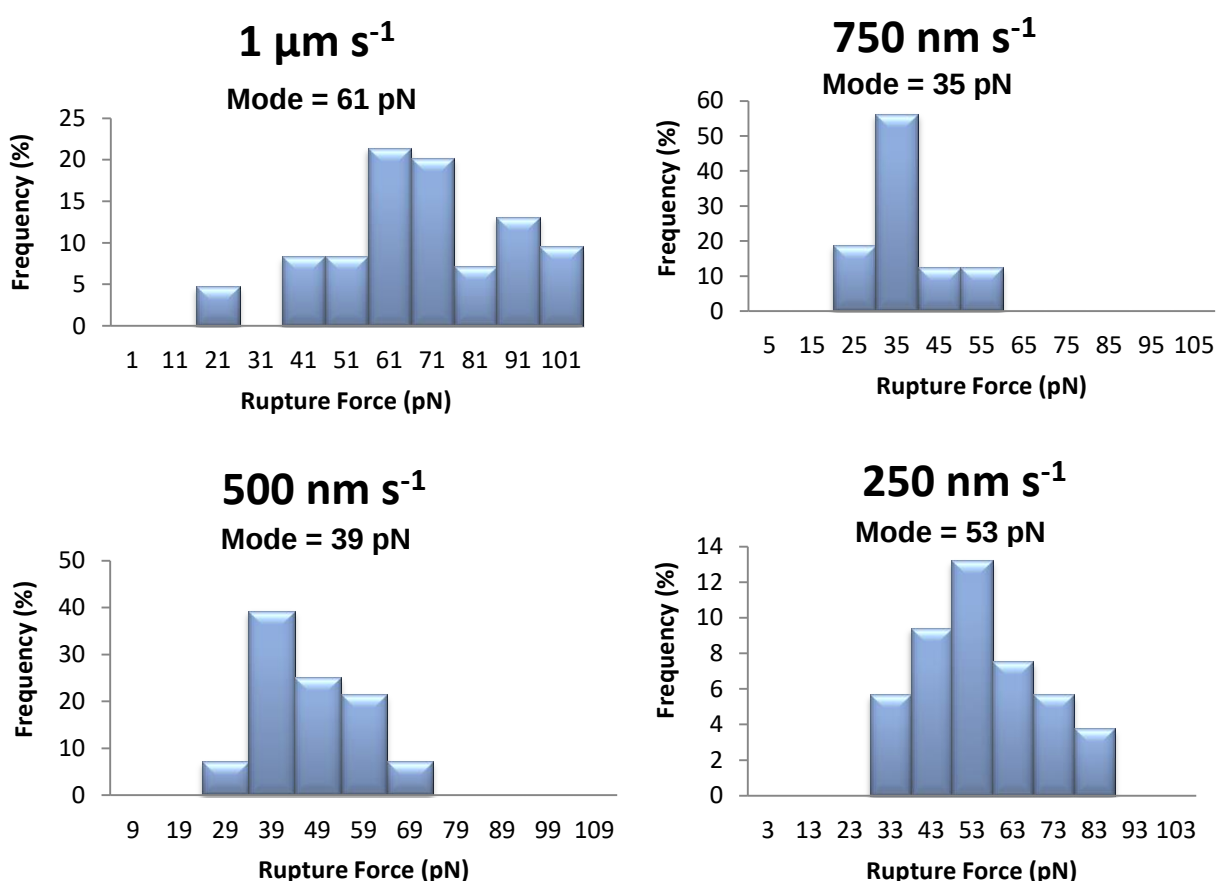


Figure 5.17. Statistical analysis of the measured bond rupture forces measured between an N-cadherin functionalized AFM tip and the surface of NP cells at tip speeds of  $1 \mu\text{m s}^{-1}$ ,  $750 \text{ nm s}^{-1}$ ,  $500 \text{ nm s}^{-1}$  and  $250 \text{ nm s}^{-1}$ . The bond rupture forces were fitted using the in-house developed program Fdist<sup>63, 109</sup> to determine the mode force of the distribution (see Chapter II, Section 2.3.). The data was then plotted as a histogram with a bin size of 10 pN to show the distribution of the specific rupture force magnitudes centred around the mode force.

Similar to E-cadherin on mES cells, these results demonstrate once more a potential discrepancy between bond rupture force magnitude and expression level of cadherins (see Section 5.3.6.). Contrary to E-cadherin, the role of N-cadherin in neural development has been studied extensively <sup>165, 169</sup>. In synapses of the central nervous system, presynaptic to postsynaptic adhesion is mediated at least in part by N-cadherins <sup>169</sup>. The results of our IHC staining presented in Chapter IV support the idea that as pluripotent mES cells differentiate towards a neuronal lineage, N-cadherin expression levels increase, and E-cadherin expression becomes less prominent.

Despite this, the N-cadherin bond rupture forces recorded here were found to be similar on NP cells compared to undifferentiated mES cells (see Section 5.3.8. below). While this finding should be treated with caution due to the limited number of repeats, it could be attributed to the fact that the neuronal precursors might have been in a premature stage at the time of the measurement, in which they did not yet possess the fully developed phenotype of fully differentiated glutamatergic neurons and hence the expression levels of N-cadherin could have been lower than expected.

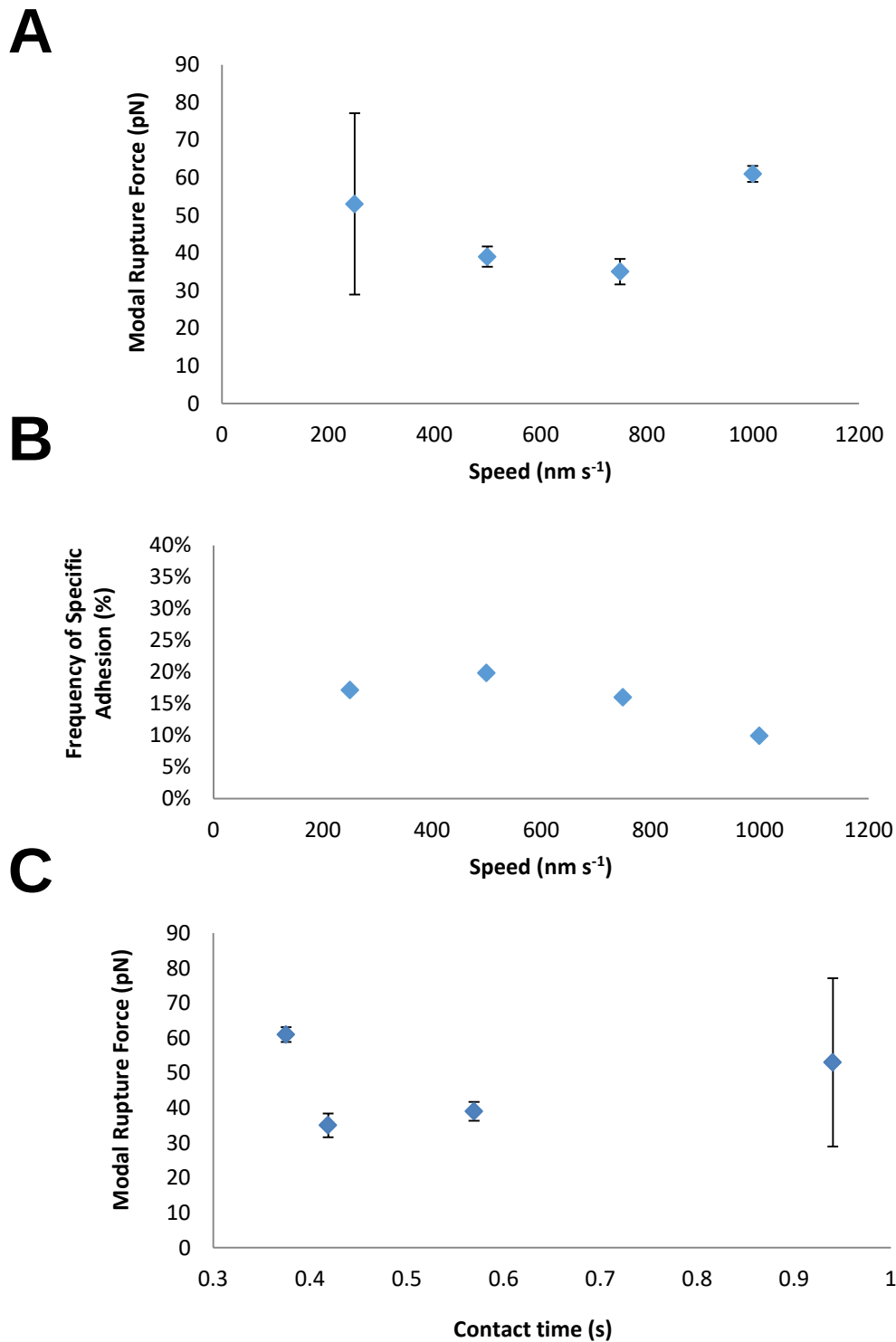


**Figure 5.18.** (A) Rupture forces of N-cadherin bonds on NP cells as a function of the AFM tip speed. Force measurements were carried out at tip speeds adjusted to the cell experiments. ( $1 \mu\text{ms}^{-1}$ ,  $750 \text{ nms}^{-1}$ ,  $500 \text{ nms}^{-1}$ ,  $250 \text{ nms}^{-1}$ ). Tip-surface interactions were measured and recorded 500 times at three different positions over a distance of  $2 \mu\text{m}$  from the cell surface for one AFM tip. As the tip speed was increased N-cadherin bond rupture forces decreased in a negative linear fashion except for  $1 \mu\text{ms}^{-1}$ . (B) The relative frequency at which specific binding between N-cadherin on the AFM tip and on the cell surface occurred showed no significant change in response to changing tip speeds and stayed between 9% and 20% (C) N-cadherin bond strength increased as the contact time was increased but was high for the lowest contact time at 0.37 seconds. Error bars indicate the standard deviation of the modal value of recorded forces.

### **5.3.8. Summary II: Combining SMFS and CFS on mES cells and NP cells**

By combining the results of SMFS and CFS on undifferentiated mES cells and NP cells it was possible to gain additional insight into the involvement of cadherin bonds in mediating cell adhesion. Figure 5.19 shows the results of the SMFS and CFS experiments for mES and NP cells in the same graph for E-cadherin (5.19.A) and N-cadherin (5.19.B).

In both SMFS and in CFS regardless of the cell type, E- and N-cadherin bond rupture forces were found to decline with increasing AFM tip speeds. Strong similarities could also be observed when measuring the rupture forces of E- and N-cadherin bonds in SMFS experiments and on the surface of undifferentiated mES cells at different tip speeds (i.e. <10 pN difference).

Compared to E-cadherin functionalized surfaces and E-cadherin bonds formed on mES cells, in preliminary experiments the rupture forces of E-cadherin bonds on NP cells appeared to be higher for all tested tip speeds. These results could indicate that the interaction of cadherin ectodomains on functionalized surfaces, were closer related to the state of cadherin interactions on mES cells, compared to those on NP cells given the similarity of rupture forces at the tested tip speeds. However, limited repeats of this experiment and a lack of blocking experiments meant that this observation still requires further investigation. Due to project time constraints a more thorough investigation was not possible here. Nevertheless the observed differences in cadherin rupture forces could indicate that a lineage specification of undifferentiated mES cells goes hand in hand with a change of cadherin mediated adhesion. It is also interesting to note that E-cadherin

forces were higher than N-cadherin in previous published results, providing further evidence for this theory<sup>165</sup>.

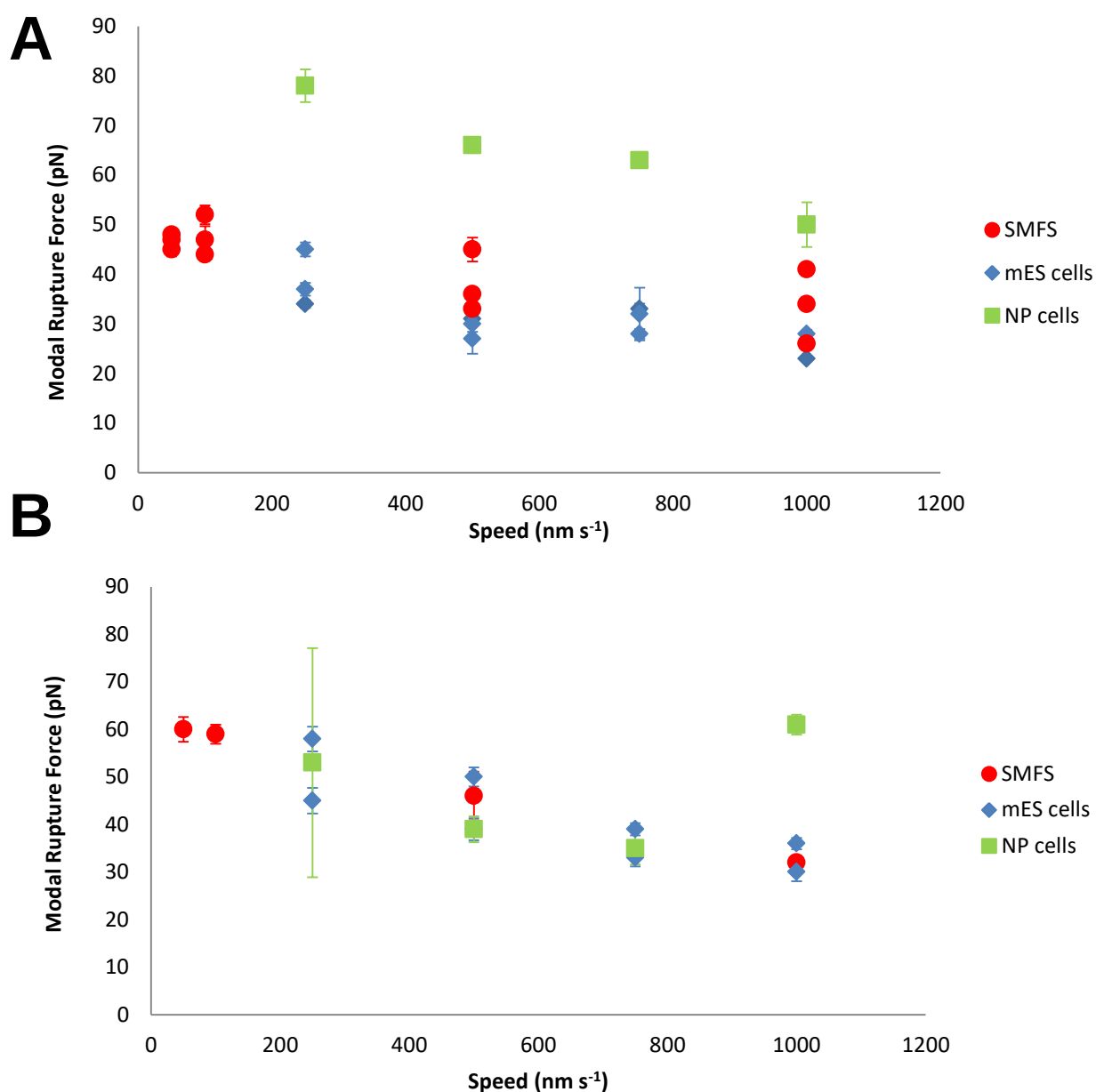


**Figure 5.19.** Comparison of the results gained in DFS studies of cadherin in SMFS and CFS on mES cells and NP cells. (A) Comparison of the results obtained for E-cadherin. (B) Corresponding results obtained for N-cadherin. The highest modal bond rupture forces could be observed when E-cadherin bonds were ruptured on the surface of NP cells. Error bars shown represent the standard deviation of the modal value of recorded forces.

Cadherin activity is regulated by intracellular signalling pathways. Cadherin-mediated contacts activate Rho family GTPases, regulate the availability of  $\beta$ -catenin to participate in Wnt signalling and function in receptor tyrosine kinase

signalling<sup>170</sup>. Although the molecular organization of the cadherin/catenin complexes appear to be similar in different cell contexts, cells receive different information from different cadherins and also process the information differently depending on cell type. Possibly the role of E-cadherin in the early developing nervous system is to maintain stable arrangements of neurons rather than to facilitate dendrite growth and cell migration. This could be the reason why E-cadherin receptors may form stronger homophillic bonds between one NP cell with another, compared to homophillic N-cadherin bonds.

## **5.4. Conclusions**

The CFS experiments could demonstrate that it was possible, for the first time, to measure bond rupture forces of E- and N-cadherin on the cell surface of undifferentiated mES cells. Recombinant cadherin constructs immobilised on AFM tips were able to form molecular bonds with native cadherin on cell surfaces. Interactions of cadherin constructs and native cadherin on the cell surface could also be blocked through administration of an antibody specific for the cadherin type on the AFM tip, this provided evidence for the authenticity of the molecular bonds investigated with the AFM and suggested that the interactions of cadherins occurred in a homophillic, specific manner. No substantial differences in modal bond rupture force or bond frequency could be observed between E- cadherin or N-cadherin bonds on functionalized surfaces or on mES cells in response to externally applied force. These findings open the door to a screening environment in which it is possible to test potential therapeutic agents that can influence cadherin interactions.

In preliminary experiments it was possible also to detect potential differences in cadherin bond formations when mES cells were allowed to differentiate into precursor cells of the neuronal lineage. Although there is limited data to support our findings on NP cells, there are indications that a lineage specification of undifferentiated mES cells goes hand in hand with a change of E-cadherin mediated adhesion. These potential changes are realized not only through changes in cadherin expression levels but also in E-cadherin bond strength.

Such a discrepancy between protein expression level and cadherin bond rupture force also highlights the usefulness of AFM in characterizing cadherin interactions. Quantitative data acquired through CFS provides an important augmentation to qualitative information gained by methods based on fluorescence probes, gene sequencing or deletion assays. The AFM data for each cell type can be considered a unique 'force signature' for the particular cell type in question. Therefore AFM could serve as a tool to identify the undifferentiated or pluripotent state of stem cells as well as to help define the health and normal function of other cell types.

# **Chapter VI**

## **Investigating cadherin interactions using holographic optical tweezers (HOT)**

### **6.1. Introduction**

Having investigated cadherin interactions on undifferentiated mES cells using AFM, an additional approach of force spectroscopy was developed as part of the work for this thesis. To study cadherin interactions at the level of single cells, a model system was tested that was based on holographic optical laser tweezers (HOT). Optical tweezers (OT) use the radiation pressure from a focused laser beam to capture and manipulate microscopic objects. These highly sensitive instruments have also been used to measure and apply forces in the range of 0.1 pN to 100 pN and to measure displacements in the nanometre range of objects ranging in size from 10 nm to over 100 nm<sup>95</sup>. For this project, HOT held great potential to explore the nature of cadherin interactions on mES cells, with a greater force detection resolution compared to AFM.

#### **6.1.1. Optical tweezers (OT) in stem cell research**

Over the last 20 years optical tweezers have become an important tool in biology<sup>171-177</sup>. Optical tweezers are now being used in the investigation of an increasing number of biochemical and biophysical processes; from measuring the basic mechanical properties of biological polymers, to understanding the

properties of the multitude of molecular motors <sup>171</sup> and receptor–ligand interactions <sup>178</sup> that drive the internal and external dynamics of individual cells <sup>177</sup>. While applications to date of optical tweezers technology have focused on determining the biophysical properties of various mammalian cells <sup>90</sup> as well as, yeast <sup>177</sup>, bacteria <sup>179</sup> and plant cells <sup>180</sup> the study of embryonic stem cells is only now coming into focus.

OT have the potential to make a meaningful contribution to the field of stem cell research by supplying much needed information on biomechanical aspects of stem cell differentiation at the single cell level, information that is hard to obtain using traditional methods like cell staining or microfluidics which rely on large numbers of cells <sup>181</sup>. To illustrate how OT can be used to advance stem cell research five example studies will be now be briefly outlined below.

Leach *et al* demonstrated that live mES could be manipulated and arranged with HOT into defined patterns and survive for at least 18 hours <sup>182</sup> (Fig.6.1.).

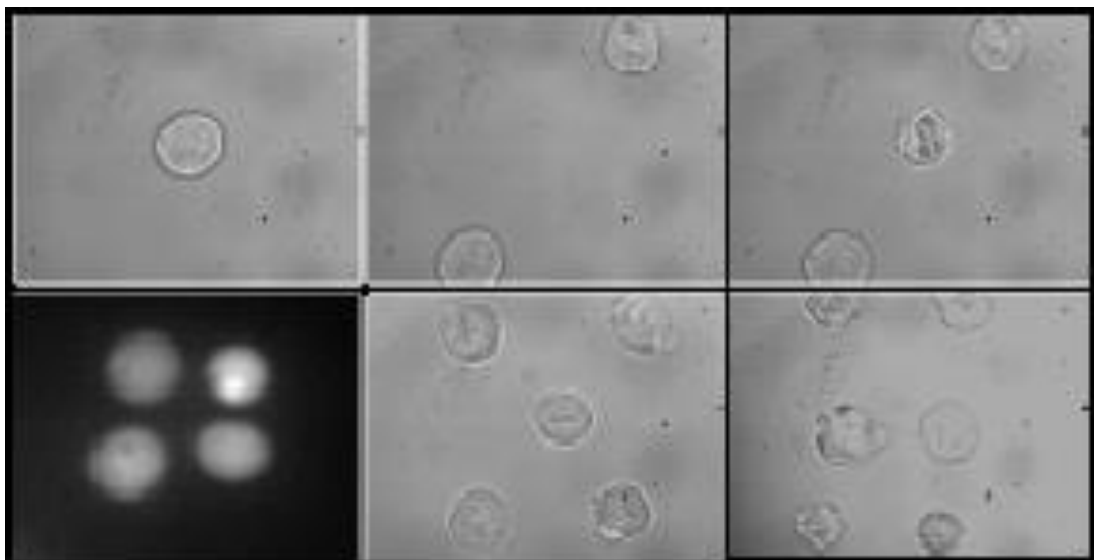


**Figure 6.1. Mouse embryonic stem (mES) cells artificially arranged into specific patterns using HOT (here shown as the six faces of a dice with the numbers being mES cells) <sup>182</sup>. The image on the lower left side shows the cells are alive via a live/dead fluorescence stain.**

By taking advantage of the ability of HOT to create multiple traps which can be moved independently (See Chapter II, Section 2.4.1.), cells could also be placed on top of each other in 3D cell construct- like configurations. The authors proposed that application of optical trapping had the potential to facilitate studies aimed to describe the impact of arrangement and position on stem cell growth and differentiation.

In similar study by Glen Kirkham *et al.* HOT were used to create bespoke mES cell micro-structures in 3D that were stabilized using hydrogels and cell-cell adhesion methods (Fig.6.2.)<sup>182</sup>. By introducing controllable and tailored structural elements made of poly(lactic-co-glycolic acid) or PLGA at precise positions during construction of these structures the controlled temporal release of chemical factors that influenced local cell behaviour could also be achieved.

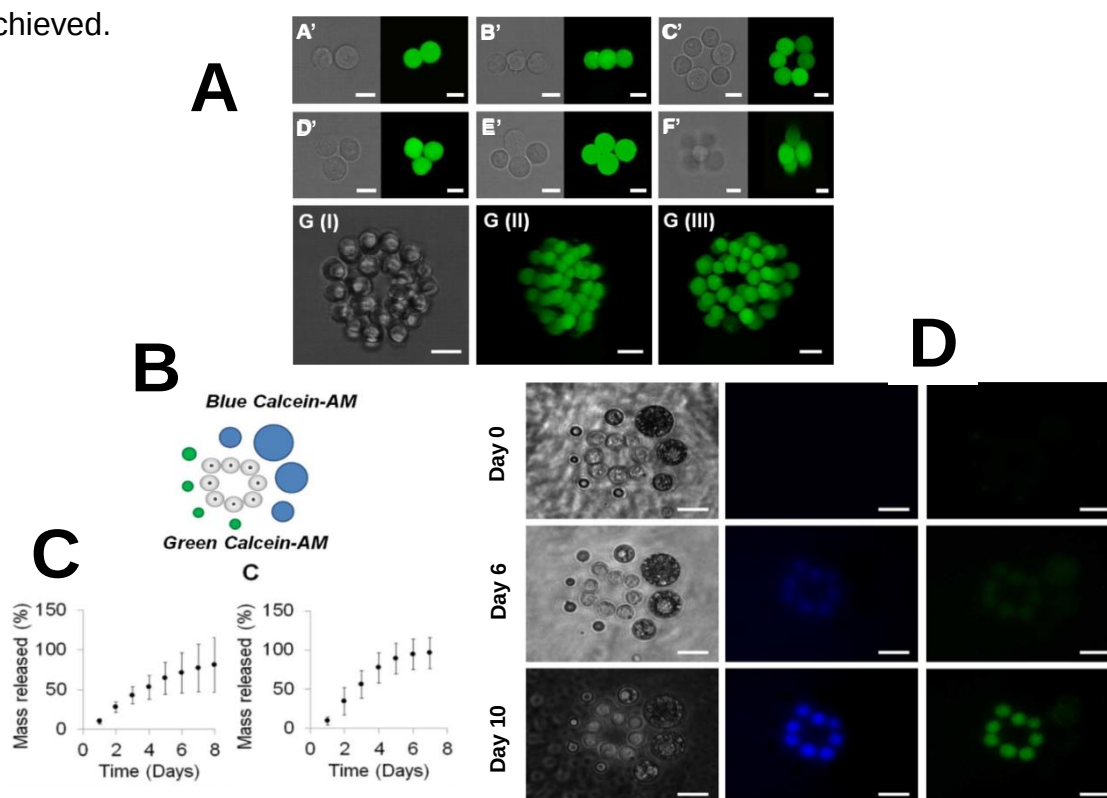


Figure 6.2. (A) Images of mES cells patterned into precise 3D structures using HOT<sup>182</sup>. The resulting architectures are stabilized using either an avidin-biotin method of cell adhesion (A'–F') or a PEG based modular hydrogel matrix (G (I)–G (III)). Images were taken in brightfield and using fluorescent confocal microscopy (green labelled images). (B) Schematic of controlled release of green and blue labelled Calcein-AM from PLGA microspheres positioned at precise locations around mES cells within agarose gels and cultured over 10 days. (C) Release profiles for green Calcein-AM microparticles. (D) Bright field and fluorescent images of resulting cultures at zero, six and ten days after pattern formation. Scale bars=12  $\mu$ m.

Tan *et al.* also utilized OT to probe mechanobiological properties of hESC by attaching two polystyrene beads on either side of a stem cell and applying laser tweezer traps on both beads in order to stretch the cell, and in this way investigate strain-related responses of uncommitted hESC and cardiomyocyte progenitor cells (Fig.6.3.)<sup>34</sup>.

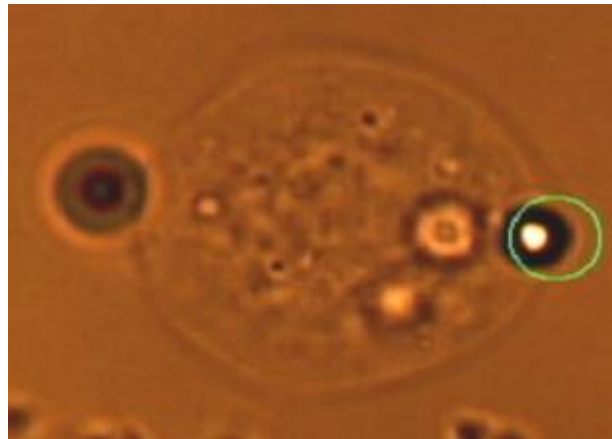


**Figure 6.3. Cell stretching of hESC and hESC-derived cardiomyocytes<sup>34</sup>. Beads were attached to the cell membrane, trapped with HOT and pulled apart.**

The stretching of the cell was accomplished by holding the cell with one bead in the same place with one optical trap while at the same time using another optical trap to move the other bead. The stretching was perpetuated until the moving bead escaped the trap and the cell returned to its original shape. It was found that committed cardiomyocyte progenitor cells possessed an overall higher degree of stiffness compared to uncommitted hESCs. It was proposed that the stiffness probing experiments could lay the foundation of modelling and understanding contractile and mechanical defects such as heart failure and muscular dystrophy.

In a study by Chen *et al.* holographic optical tweezers (HOT) were employed to study cell fusion of hESCs in a combination approach of HOT with optical

scissors, the latter being a pulsed UV laser that is used to cut the cell membrane of two human embryonic stem cells at the point of contact between them in order to initiate the fusion of these cells <sup>183</sup> (Fig.6.4.).

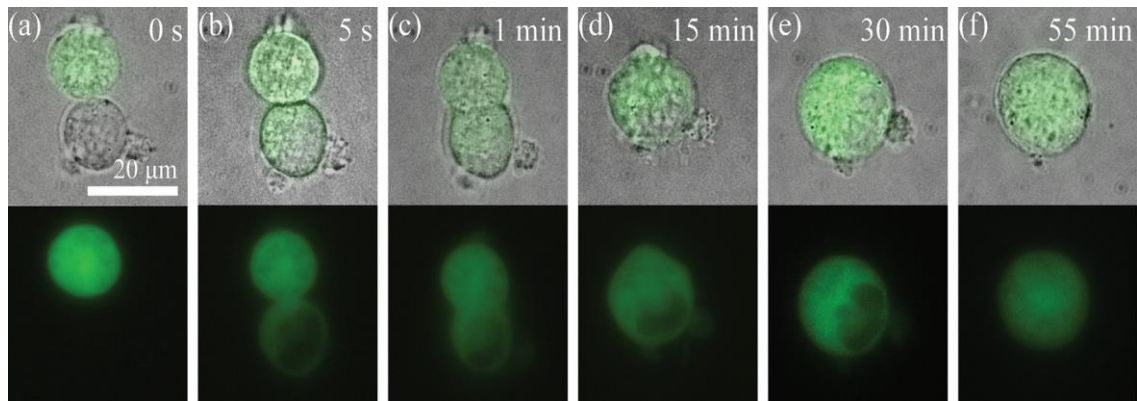


**Figure 6.4. Laser-induced cell fusion of two hESCs in suspension <sup>183</sup>.** (a) A GFP labelled cell (green) and an unlabelled cell (transparent) formed a cell pair before laser cutting. (b) After laser cutting, GFP transfer from the green cell to the transparent cell occurred. (c)–(f) Time lapse images showing the fusion process (c) 1 min, (d) 15min, (e) 30 min, and (f) 55 min after laser cutting.

Cell fusion is a natural process and occurs during embryogenesis and morphogenesis as part of tissue formation and differentiation of muscle and trophoblast cells <sup>184</sup>. In the experiment, hESC were dissociated enzymatically then grouped into two groups. One group was labelled with green fluorescent protein (GFP) while the other group was left unlabelled. The two groups were mixed and one GFP labelled cell and one unlabelled cell were trapped and manipulated by OT to form a cell pair and moved towards a defined spot for laser scissor cutting. The membrane was cut using five pulses of the UV laser to avoid unnecessary cell damage. When the membranes were cut at the point of contact, cytoplasmic GFP from labelled cells flowed into the unlabelled cells which indicated the beginning of cell fusion. The contact point got larger and formed into a tunnel connecting the two cells. Gradually the two

spherical cells formed one elliptical cell as their cytoplasms continued to mix. This process continued until the labelled and unlabelled cell formed a single spherical synkaryon, which is a cell that has a nucleus created by fusion of one cell's nucleus with the nucleus of another cell.

Titushkin and his colleagues worked with OT to determine distinct membrane mechanical properties of human mesenchymal stem cells by extracting tethers out of the outer cell membrane <sup>185</sup> (Fig.6.5.). Tethers are part of the cell membrane which form when the cell membrane is being dragged towards one specific direction and becomes detached from the cytoskeleton in a localized position. The cell membrane then assumes the configuration of a long thin cylindrical, hollow shape, known as the tether. The tether properties are believed to be crucial for many of the biological functions that the cell membrane has in proliferation, differentiation and mitosis such as maintenance of membrane electromechanical forces and upkeep of the membrane reservoir. The membrane reservoir is the concept that the cell is able to keep the membrane tension constant by compensating for mechanical and chemical perturbations occurring during morphological changes and tissue homeostasis.

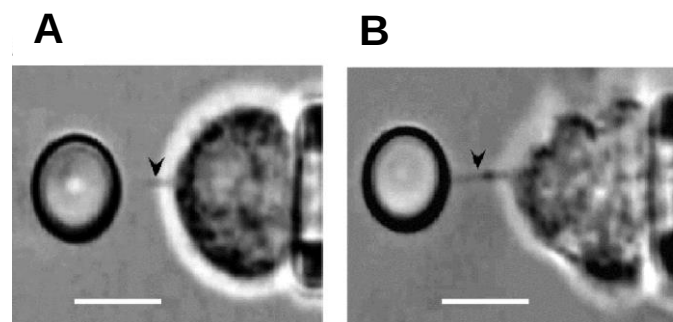


Figure 6.5. Principle of tether extraction <sup>186</sup>. This technique allows the study of membrane reservoirs of stem cells using OT. (A) Functionalised beads are attached to the cell surface and tethers are extracted. (B) Eventually the membrane potential is exhausted. The black arrow indicates the membrane tether. These experiments provide information on the mechanical characteristics of the stem cell membrane in response to environmental cues e.g. connectedness with other cells and interaction with the cytoskeleton. The scale bars in both (A) and (B) represent five  $\mu\text{m}$ .

However, if the mechanical or chemical strain on the plasma membrane exceeds a certain threshold the membrane reservoir can be depleted which means that the cell membrane experiences an abrupt rise in membrane tension and can no longer cope with any kind of morphological change.

Membrane tether extraction has been established in multiple studies as the most accurate method to quantitatively and selectively measure the membrane reservoir <sup>185-187</sup>. In many of these studies OT are used to trap functionalized beads which are coated with biological active molecules such as antibodies or extracellular matrix proteins and attached to the cell surface. A membrane tether is formed by pulling the membrane attached bead away from the cell and thereby detaching the cell membrane from the cytoskeleton in a specific location forcing the cell to use up its entire membrane reservoir capacity. By quantifying the tether length and the force required to form the tether, it is possible to analyse the cell membrane's mechanical characteristics in response to environmental cues such as the degree of connectivity with other cells and interaction with the cytoskeleton.

All of the above mentioned studies demonstrate how OT can be used as a highly sensitive force detection and manipulation tool capable of furthering stem cell research. However, as yet, OT have been used in very few studies to probe cell – cell interactions in embryonic development. The aim of the work described in this chapter was to use the HOT set up described in Chapter II, Section 2.4.1., to quantify adhesion forces between the cell membrane protein E- cadherin on the surface of undifferentiated mES cells with high force resolution.

Precise force measurements depend on accurately measuring the stiffness of the optical trap itself <sup>187-189</sup>. The trap stiffness depends on many different factors such as total applied laser power and the number of traps formed in the experiment. Therefore, before performing measurements on cells it was first necessary to test the impact of each of these factors on the quality of the optical trap.

## 6.2. Materials and Methods

### 6.2.1. Trap stiffness measurement

In order to effectively measure and apply known forces with HOT it is necessary to determine the stiffness of the trap. The optical trap is treated as a Hookian spring with harmonic potential. The stiffness  $k$  of the optical trap is given by:

$$k = \frac{k_B T}{\langle x^2 \rangle}$$

Equation 6.1.

Where  $k_B$  is the Boltzman constant,  $T$  is the temperature and  $\langle x^2 \rangle$  the time-averaged positional variance of the trapped bead. By using similar principles the quality of the created trap can be further described by making viscosity measurements, of liquid samples of known viscosity <sup>187</sup>.

### 6.2.2 Viscosity measurements with HOT

When measured with the optical trap the viscosity  $\eta$  of the solution in which dielectric beads are suspended is given by:

$$\eta = \frac{\tau k_B T}{6\pi r \langle x^2 \rangle}$$

Equation 6.2.

Where the  $\tau$  is the decay time of the tracked particle normalized position autocorrelation <sup>188, 189</sup>.

This theoretical framework was applied within this chapter to make trap stiffness and viscosity measurements in reference glycerol solutions of different concentrations, as a function of laser power. To this end individual dielectric microspheres were trapped and the customized analysis software (written in LabView) used to measure stiffness and viscosity based on the equations and definitions detailed above.

### **6.2.3. Characterizing the quality of the optical trap: Experimental procedure for trap stiffness and viscosity measurements**

Non-porous silica microspheres (Bangs Laboratories, 2  $\mu\text{m}$  diameter) were mixed with pure water and water-glycerol mixtures. Samples were prepared by re-suspending 0.25  $\mu\text{L}$  of bead solution in 5 mL  $\text{H}_2\text{O}$  and then diluting by 1:40 with the respective glycerol mixture. Bead concentrations were empirically determined as part of the original work of this thesis and iterative investigation was necessary to determine the optimal ratios of the bead solutions. If the bead concentration was too high randomly floating beads would knock the trapped bead out of the optical trap whereas when the concentration was too low it became difficult to locate beads in solution.

The bead suspension was loaded onto a glass microscope slide and sealed by gluing a cover glass on top of the sample area. The sample slide was mounted on the motorized X-Y microscope stage and the beads were brought into focus by adjusting the objective lens (see HOT set up, Chapter II, Section 2.4.1.). The CMOS camera produced a live image-feed which was recorded

and analysed using particle tracking software developed by the Padgett group (see Appendix A). The trap stiffness was measured by recording the mean squared displacement of the bead  $\langle x^2 \rangle$ .

In order to study bead movement inside the trap, a time series of position measurements on the X and Y axis was converted into a power spectral density function (see Appendix B). A trapped particle produced a characteristic shape of a particle displacement power spectrum. The amplitude of the particle motion power spectrum decreased at higher frequencies. By using the curve fitting software the low frequency intercept  $P_0$  and the corner frequency  $F_0$  were estimated. The power spectrum provided information about how much and how quickly a bead moved inside the trap. Lastly, for measurement of viscosity, the mean displacement squared  $\langle x^2 \rangle$  for a time series of particle position measurements was calculated and an autocorrelation function that compares the similarity of measurements with themselves over time gave, the characteristic decay time of the system  $\tau$ . With these two parameters the viscosity of the solution could be measured by using Equation 6.2. (see also Appendix C).

#### **6.2.4. Force measurements using HOT**

Once trap stiffness was determined, to measure forces with the HOT's, displacement of the bead (on the X axis in ( $\mu\text{m}$ )) within the optical trap needed to be converted to a force value. This was done by monitoring the change in position of the bead inside the trap as shown in Fig.6.6.A. Figure 6.6.B shows the movement of the bead inside the trap in X and Y direction inside the trap. This movement was then converted using Hooke's law to get the force in X

and Y plane of the optical trap necessary to keep the bead inside the trap (see Appendix D). To measure adhesive forces between beads this measurement was performed as another bead was moved into and out of contact with the stationary bead.

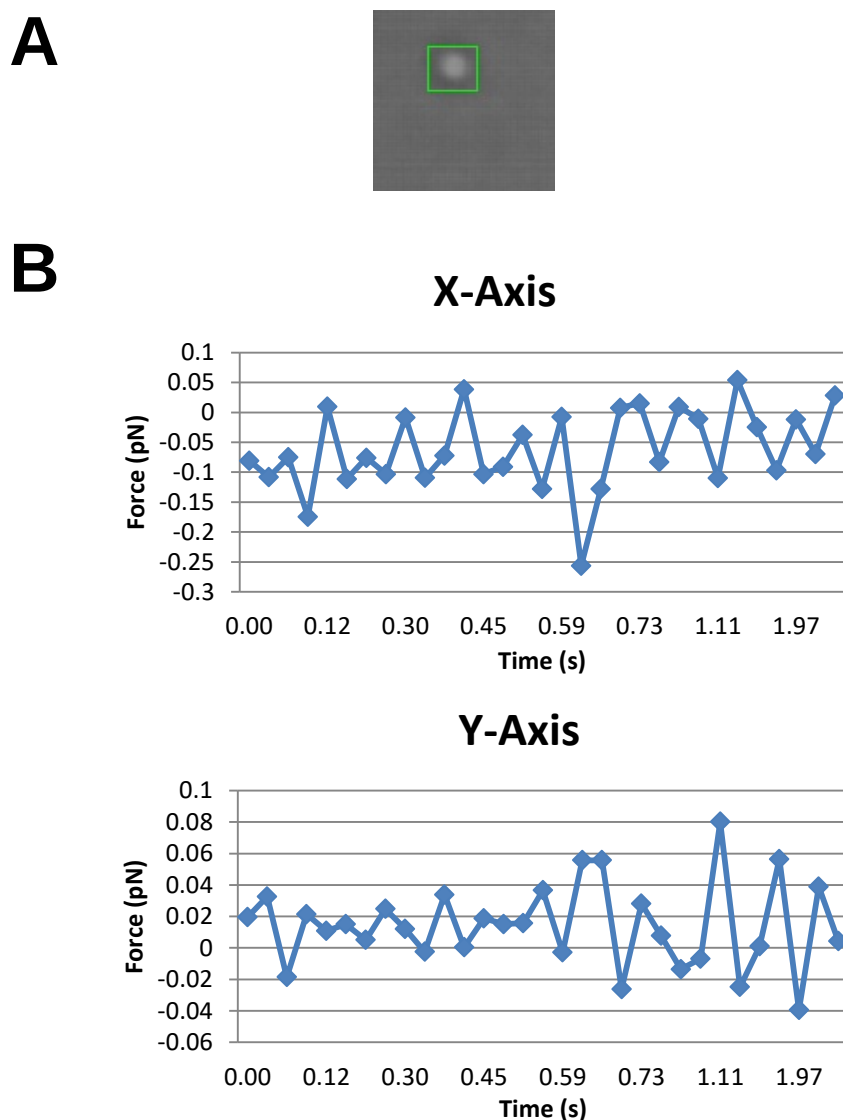


**Figure 6.6.** Force measurements with the HOT. (A) Microscopic view of a bead that is trapped and brought into the region of interest (ROI) and traced by the camera. (B) Force profile of a bead moving inside the optical trap in X (horizontal) and Y (vertical) direction.

### **6.2.5. Functionalization of silica beads with E-cadherin ectodomains**

Beads used for HOT experiments were functionalized using the silanization protocol developed in the laboratory of Prof. E. Evans (University of British Columbia, Vancouver, Canada). Non-porous silica microspheres (Bangs Laboratories, 2  $\mu\text{m}$  diameter) were cleaned by adding the 500  $\mu\text{L}$  of bead suspension to boiling  $\text{H}_2\text{O}_2/\text{NH}_4\text{OH}$  (hydrogen peroxide /ammonium hydroxide or basic piranha) solution for five minutes. The beads were then washed five times by centrifugation (3500 g for five minutes) and stored at 4°C overnight. The beads were silanized by covalently attaching thiol (SH) groups to the bead surface. This was done by mixing the beads in a mixture containing acetic acid (Sigma-Aldrich), methanol (Sigma-Aldrich), and MPTMS (3-mercaptopropyl)trimethoxysilane) (Sigma-Aldrich) for three hours at room temperature in a rotary mixer. The beads were then washed once in methanol and dried for five minutes at 120°C in a pre-heated drying oven and afterwards further dried in a vacuum desiccator filled with silica gel overnight. The AMAS crosslinker (Thermo Scientific) was then attached to the silanized beads via their thiol groups by incubating the beads in AMAS in DMSO for one hour at room temperature in a centrifuge tube (Eppendorf), with gentle end to end mixing. Finally the beads were washed once in carbonate-bicarbonate buffer (pH 8.5)(Sigma) and incubated for three hours at room temperature with gentle, occasional manual mixing in a carbonate-bicarbonate buffer solution containing the E-cadherin ectodomain construct in a concentration of 10  $\mu\text{g}/\text{mL}$  of protein for 0.5 mg of beads. After a final

washing step in carbonate-bicarbonate buffer the beads were resuspended in PBS and kept at 4°C until use.

## **6.3. Results and Discussion**

### **6.3.1. Impact of laser power on bead movement, trap stiffness and viscosity measurements**

The ability of the HOT to adjust the trap stiffness by adjusting the applied laser power in real-time, during the measurement, makes them especially well-suited to make high resolution mechanical measurements on individual biological molecules. The stiffness of an optical trap should increase by increasing the laser power of the trap <sup>190</sup>. Therefore to investigate this relationship for the HOT system, trap stiffness (recorded in H<sub>2</sub>O) as a function of the applied laser power, was measured at 20% intervals of increasing laser power.

Beads were trapped with the HOT and the respective software used to monitor the trap stiffness (see Appendix A and B). As can be seen in Figure 6.7.A the stiffness had a strong positive correlation with the laser power, which means that the stiffness of the trap increased as the laser power increased. The data in Figure 6.7. represents the stiffness measured in one specific location in the focal plane, for three different beads. The motion of the bead inside the trap is related to the strength of the trap: as the trap stiffness increases, the frequency with which the bead moves inside the trap increases as well.

As trap stiffness increases the corner frequency of the power spectral density function of the particle tracking software increases and can serve as an additional means to determine the stiffness of the trap (see Appendix B) <sup>141</sup>.

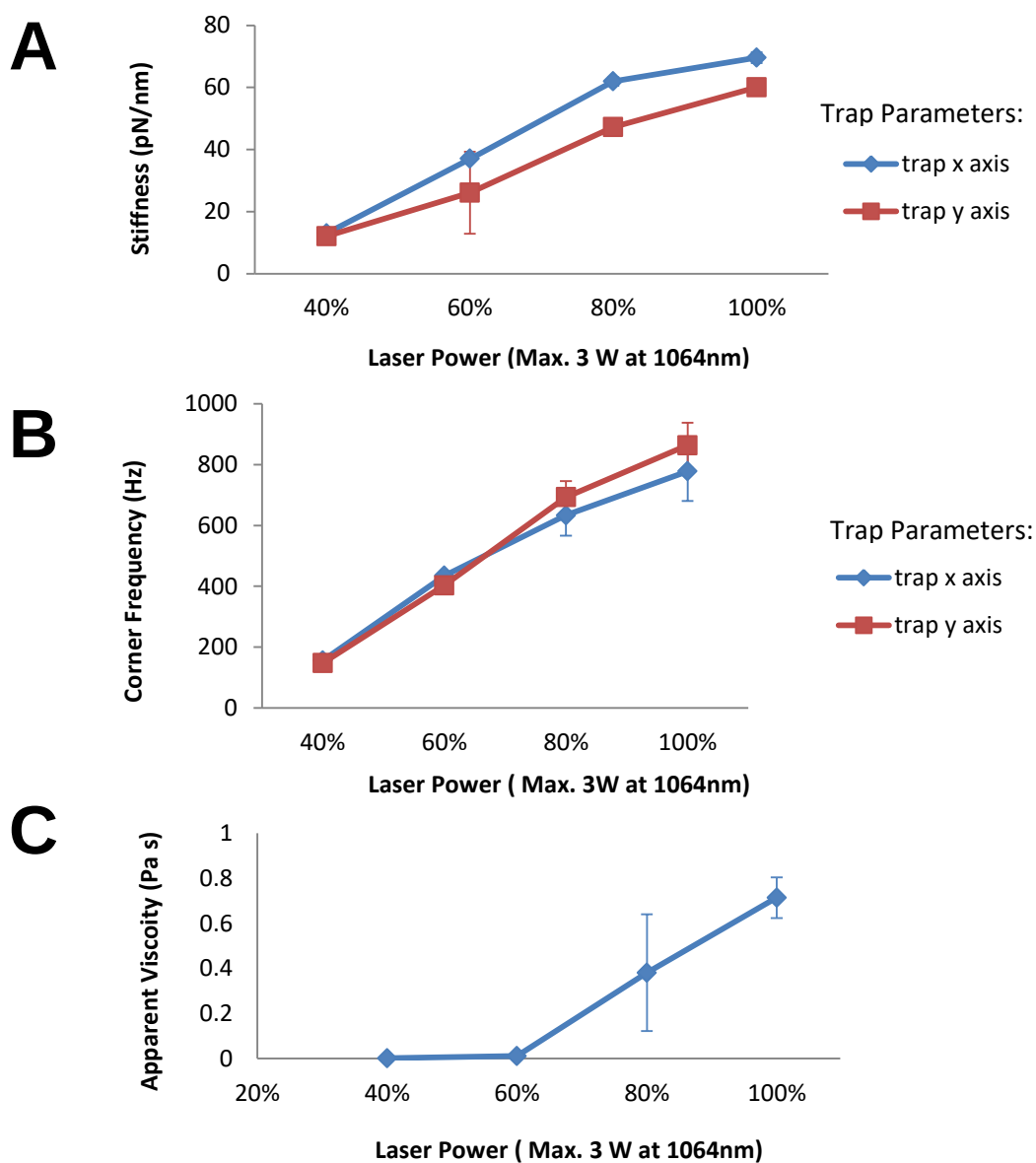


Figure 6.7. Impact of laser power on trap stiffness. (A) Stiffness increased proportionately with increasing laser power. (B) The corner frequency also increased proportionately with increasing laser power. (C) Viscosity measurements of pure water in response to increasing laser power. With increasing laser power, the measurement itself becomes increasingly more inaccurate.

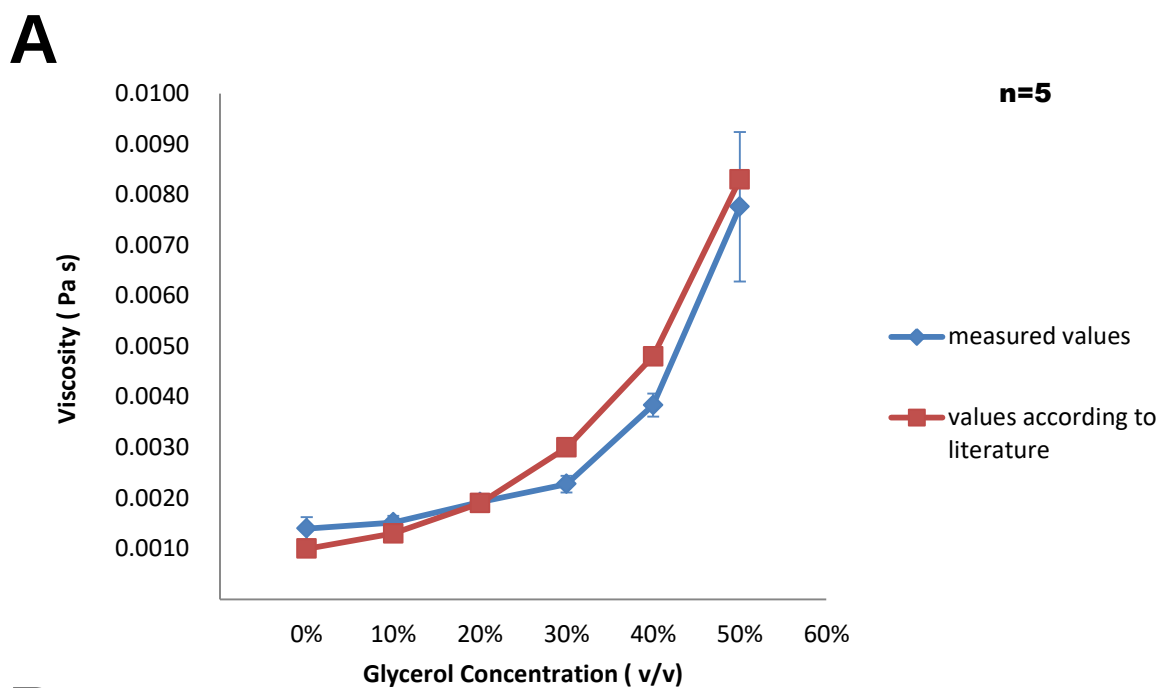
As shown in Figure 6.7.B the corner frequency also increased proportionately with increasing laser power. Figure 6.7.C shows that as viscosity measurements, (calculated from the corner frequency and made in pure water samples) are made as laser power is increased, the measurement itself becomes increasingly more inaccurate. This is highlighted by an apparent increase in recorded viscosity, although the actual viscosity in the H<sub>2</sub>O sample was not changed. This variation began to occur at laser powers larger than 60% and points to the fact that as the trap becomes stiffer and the beads inside the trap travel short distances more quickly, they are more likely move at or beyond the sampling limits of the camera.

The auto correlation software, used to determine the viscosity, tracks the position of the particle in time and relates it to its previous position and interprets this change in variance as change in the viscosity e.g. due to a shorter movement to time correlation. Apparently between 40% and 60% of the applied laser power there appears to be a 'safe zone' where the described effects do not yet have a strong enough effect to perturb the viscosity measurements. These results suggested that special care needed to be taken when using laser powers above 40% in experiments that involve working with pure water samples, to avoid instrument related errors.

### **6.3.2. Viscosity measurements using HOT**

To further explore the ability of the HOT set up to perform accurate measurements, the viscosity of several glycerol standards was measured at a laser power of 40%. As can be seen in Figure 6.8.A and 6.8.B, viscosity measurements that were made with the HOT by trapping five different beads

in each glycerol concentration, were in close agreement with viscosity values for glycerol solutions obtained from literature <sup>191</sup>. It is worth noting that the viscosity measurements when taken with HOT were found to have a minor deviation of  $\pm 0.009 \text{ Pa s}$  which is likely due to miniscule differences in radius between individual beads. These experiments also highlight the influence of the refractive index on viscosity measurements. For example it was not possible to trap microspheres beyond a glycerol concentration higher than 50% (v/v), because the refractive index of the solution outside the trap comes increasingly closer to that of the silica bead, and as a result the trap becomes weaker. Indeed in 50% glycerol the refractive index of the solution (1.4043) almost matches that of the bead itself (1.46). Figure 6.8.C highlights this through the appearance of water and 80% glycerol solutions under the microscope. It can be seen that the contrast in the image acquired in 80% glycerol solution is considerably reduced due to the closeness in the refractive index of the glycerol solution to the bead.



**B**

| Glycerol (v/v) | Measured values (Pa s) | Values according to literature(Pa s ) |
|----------------|------------------------|---------------------------------------|
| 0              | 0.0014±0.0002          | 0.0010                                |
| 10             | 0.0015±0.0001          | 0.0013                                |
| 20             | 0.0019±0.0001          | 0.0019                                |
| 30             | 0.0023±0.0001          | 0.0030                                |
| 40             | 0.0038±0.0002          | 0.0048                                |
| 50             | 0.0078±0.00148         | 0.0083                                |

**C**

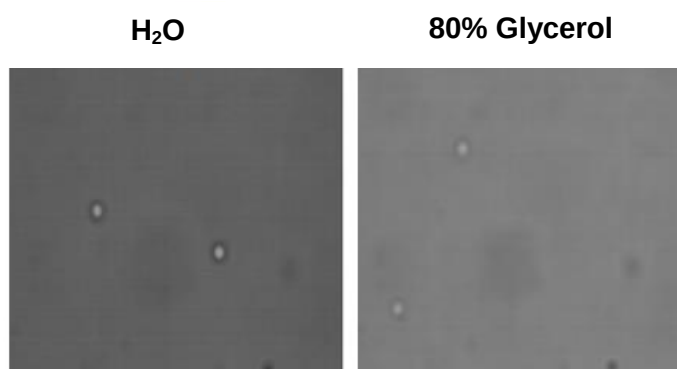


Figure 6.8. Viscosity measurements using HOT. (A) The viscosity increases with increasing glycerol concentrations. (B) Glycerol concentrations values of measured viscosity are in close agreement with those reported in literature <sup>191</sup>. (C) The different appearance of H<sub>2</sub>O and 80% glycerol samples under the microscope. In 80% glycerol the refractive indices of bead and solution are almost equal and trapping becomes impossible.

### **6.3.3. Variation in trap stiffness with spatial position within the sample plane**

To explore if there was any variation in trap stiffness as the position of the trap was altered in the focal plane, trap stiffness was measured in H<sub>2</sub>O, 30% and 50% glycerol solutions at different X and Y positions. An arbitrary central rectangle was defined in the centre of the focal plane ranging from -30  $\mu\text{m}$  to +30  $\mu\text{m}$  in the X direction and -20  $\mu\text{m}$  to +20  $\mu\text{m}$  in Y direction (see Fig.6.9.A). The red circles shown in Figure 6.9.A represent individual trap stiffness measurements obtained in X and Y axes, at defined positions across this rectangle. Large, symmetric circles represent optical traps that are stiffer and have the same stiffness in the X and Y direction. For example a large circle is seen in the centre of the schematic for H<sub>2</sub>O in Fig. 6.9.A. This position represents the zero-order position at which the diffracted laser beam is directly reflected off the in the centre of the SLM and projected onto the focal plane (see Chapter II, Section 2.4.1). The trap stiffness measurements showed that trap stiffness varied according to X and Y position in the focal plane. However, some locations were found to consistently have traps that were stiffer and more symmetric in their trap stiffness than other positions. The wave front of the first and higher-order diffracted laser beam (towards the edges of the focal plane) are modified to match the desired amplitude, frequency and phase shift. This means that the trap stiffness will vary according to the position of the diffracted light emerging from the SLM and projected onto the focal plane.

The position of (X,-30;Y,10) was identified as a good position for mechanical measurements with the HOT, since it showed high trap stiffness as well as

trap symmetry making it less prone to variations in measurements. Therefore, this position (approximately in the upper left corner of the focal plane) was chosen for subsequent measurements with the optical tweezers.

In the 30% and 50% glycerol samples the optical traps were again found to have much lower stiffnesses in the X and Y plane in every measured position compared to the water sample (Fig.6.9.). This, again, can be explained with the increase of the refractive index of the solution, which is approaching the refractive index of the bead itself (see Section 6.3.2.). As the concentration of glycerol increases the optical trap becomes increasingly weaker. At 50% glycerol concentration, the trap stiffness has decreased to an extent that the traps are hardly present.

Interestingly viscosity measurements in 30% glycerol, however, remained constant and accurate even at laser powers beyond 45% laser power (Figure 6.9.D). Only at 85% laser power did the viscosity reading begin to increase and thus to become inaccurate. These findings suggest that the increase in trap stiffness at this power was making the movements of the bead too small and quick for the camera to track movements correctly. Note the error bars at 90% and 100% are also larger.

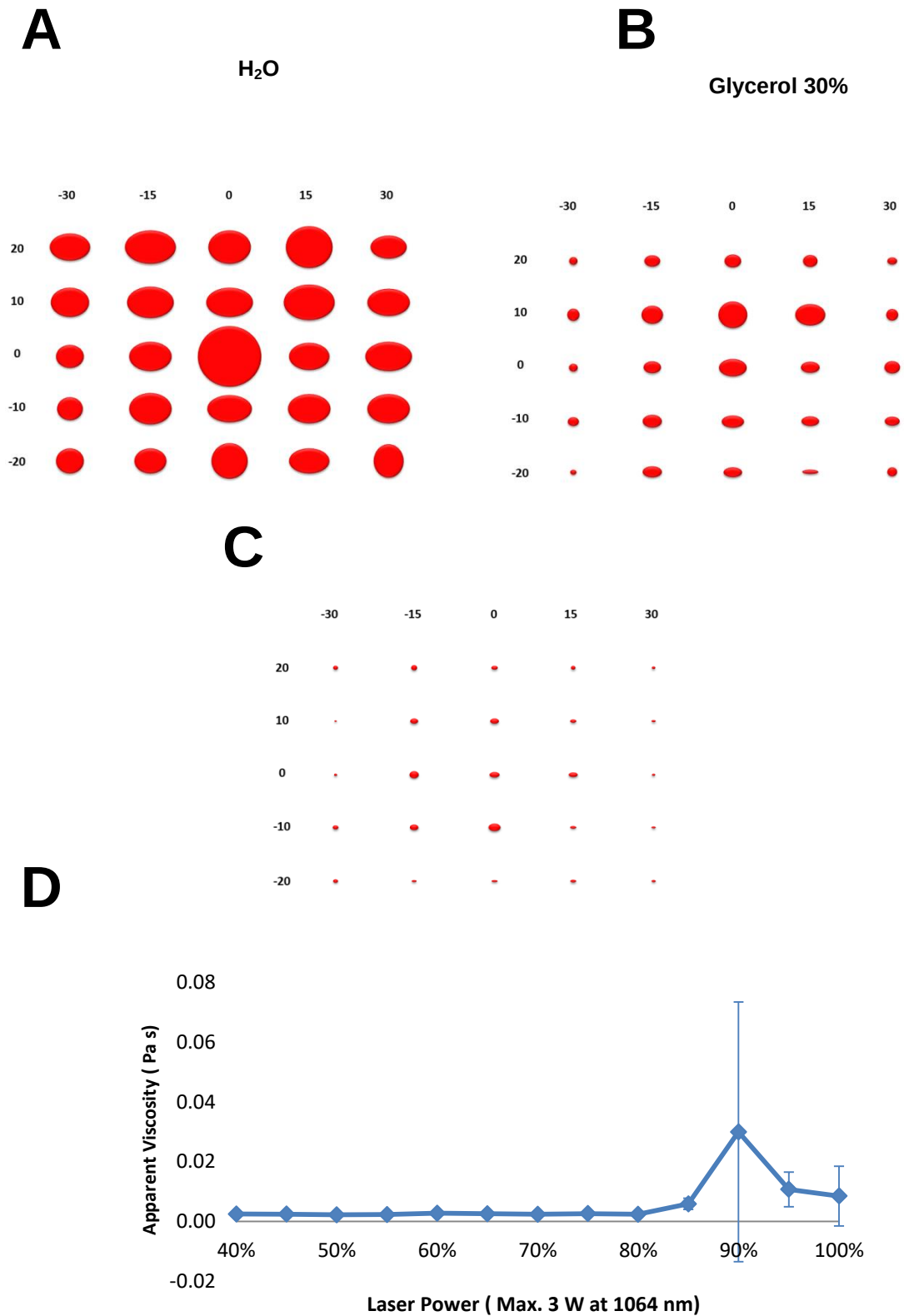


Figure 6.9. Impact of spatial position on trap stiffness. Trap stiffness at defined positions across the focal plane in(A)  $H_2O$ , (B) 30% and (C) 50% glycerol solutions. The red circles represent trap stiffness measurements in X and Y direction at each position. Trap stiffness varies with position in all three solutions. In  $H_2O$  samples the stiffness values are much higher compared to the more viscous glycerol solutions.(D) Apparent measured viscosity recorded in a 30% glycerol solution with increasing laser power. The viscosity reading remained stable until 80% laser power was applied. Only above 85% laser power did the viscosity reading begin to increase and thus to become inaccurate.

#### **6.3.4. Summary characterization results of the HOT**

In summary the measurements made with the HOT showed that precise measurements were possible with this system and that the quality of the optical trap depended on various factors such as the applied laser power, refractive index and lateral position in the focal plane. Specifically it was found that in order to provide optimal conditions for measurements, they needed to be carried out with no more than 60% of applied laser power and in a medium of refractive index of less than 1.46. To further reduce variations, force measurements needed to be recorded in a specific location in the upper left corner of the focal plane. With this knowledge it was then possible to progress to force measurements using the HOT system.

#### **6.3.5. Adhesion force measurements with HOT –A worked example**

First, the trap stiffness ( $k$ ) was measured as described in Section 6.2.4. above. To measure forces a bead was captured and its position was tracked with the trap by holding it stationary. A second bead was then trapped with a second optical trap and moved into and out of contact with the stationary bead and the position of the interacting particle monitored.

Figure 6.12.A shows the appearance of two silica beads that were trapped in this way for an adhesion force measurement under the microscope. As beads were brought into and out of contact by controlled positioning using the HOT, the force exerted on the stationary, traced bead (on the right) over time was measured. Figure 6.12.B shows the progression of this force measurement as

a force vs. time graph of the X-axis of the stationary, traced optical trap. (a) The beads move freely in their respective trap as the movable bead (on the left) is moved closer towards the stationary bead. (b) The beads jump into contact and the force necessary to hold the stationary bead in the trap increases until the movable bead is not moved further and held in contact with the stationary bead. This is indicated by the plateau in force (c). The movable left bead is then moved in the opposite direction using the HOT (d). Eventually the beads lose contact but, in this case are initially prevented from doing so by the formation of an adhesion event (e). Such an adhesion event is evident by a brief increase in force which then drops back to the base level. The magnitude of the drop indicates the force required to break the inter particle adhesion. This cycle was repeated for each force measurement. The adhesion event shown in Figure 6.12.B represents the adhesion between two touching, beads that are slowly separated and with an approximate magnitude of 200 fN.

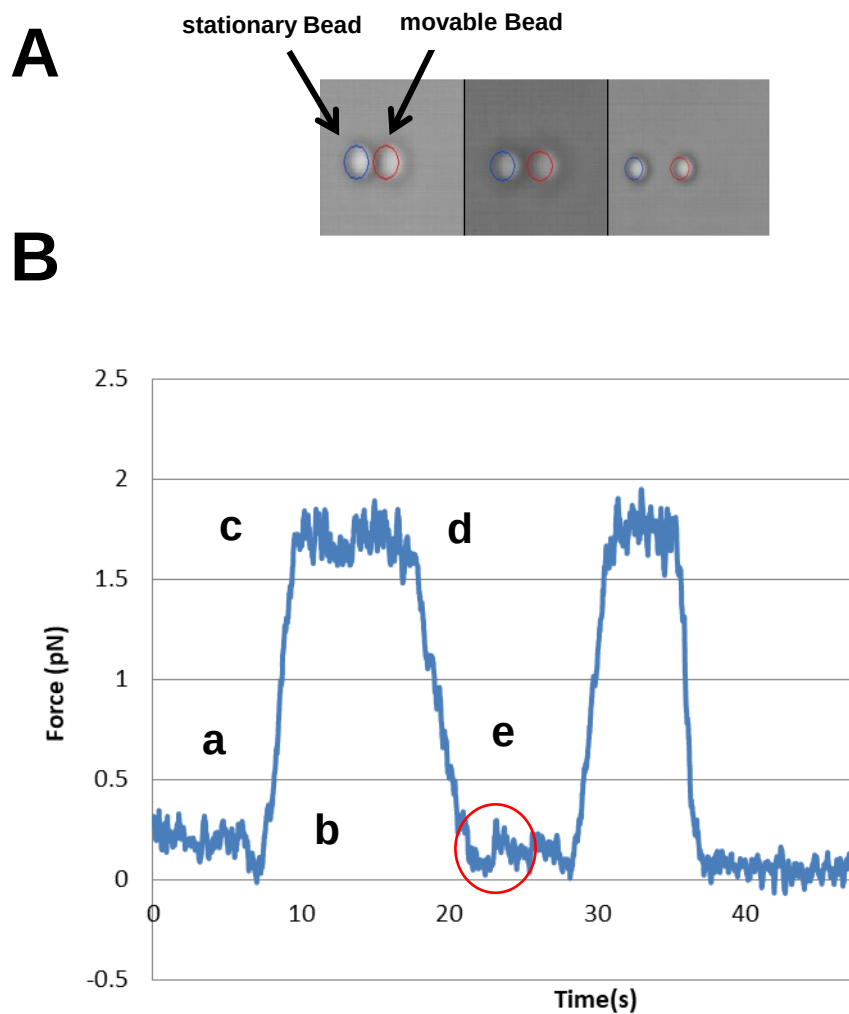


**Figure 6.10. Adhesion force measurement using the HOT. (A) Microscopic view of two beads that are trapped for a force measurement. Two beads are captured using two optical traps. The right bead is kept stationary. The left bead is moved towards the stationary bead until they make contact. The left bead is subsequently moved away from the stationary bead again. (B) Progression of a force measurement as force vs time graph of the X-axis of the stationary, traced optical trap. (a) Beads move freely inside the traps. (b) Beads jump into contact and are pressed against each other. (c) Beads are held in contact. (d) The movable left bead is retracted until it loses contact and the force returns to initial base level. (e) The red circle shows the adhesion event initially preventing the beads from separating. This adhesion event had a rupture force magnitude of 200 fN.**

### 6.3.6. E-cadherin force measurements with the HOT

Following the method detailed above, HOT were used to measure the interaction forces between uncoated silica beads or beads that were coated with E-cadherin. Figure 6.11. shows examples of force measurement traces obtained. It can be seen that compared to the ‘ideal’ measurement profile shown in Figure 6.10. many other type of traces were recorded. Before further measurements with cadherin coated beads could be made, it was therefore important to explore the origin of this variability in force measurements.

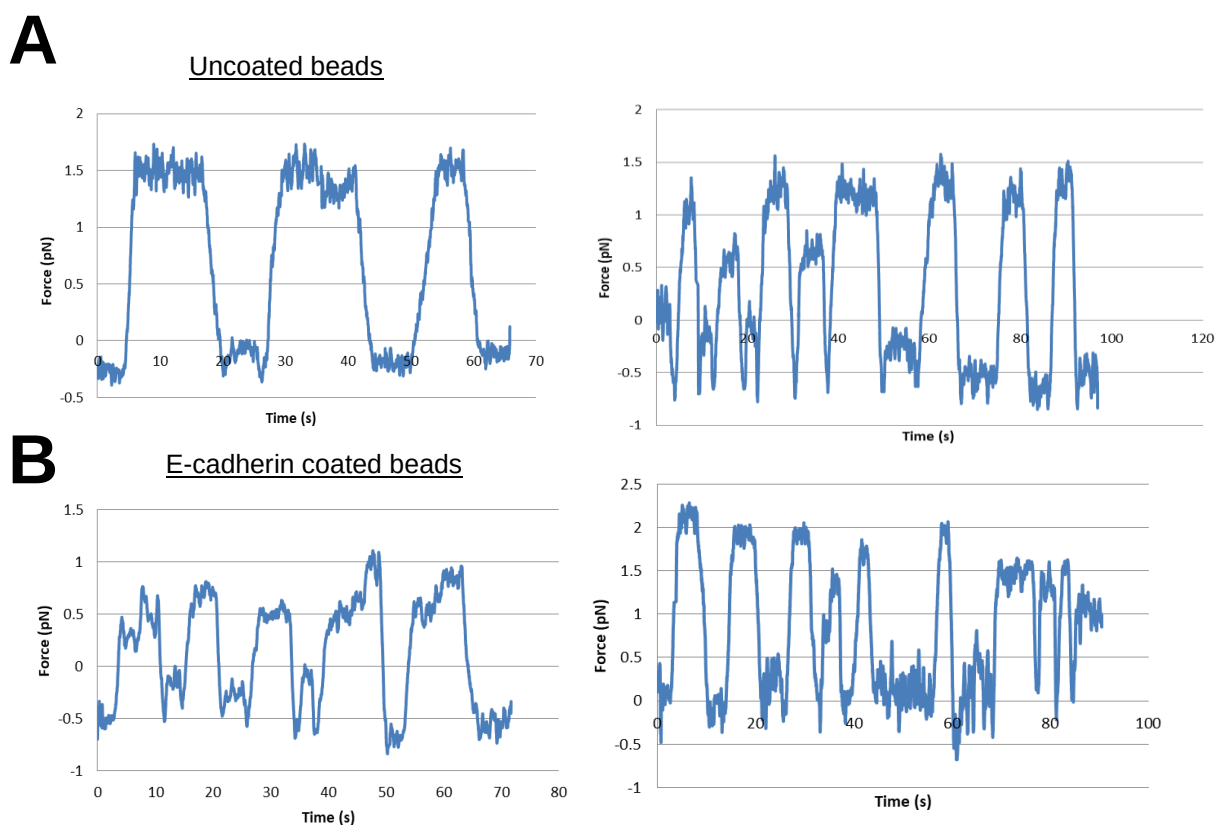


Figure 6.11. Measurements of adhesion forces between (A) uncoated beads and (B) E-cadherin coated beads by controlled contact using HOT. Each trough-peak-through cycle represents one approach-retract cycle and corresponds to one force distance (FD) curve recorded with the AFM. Although interaction forces could be measured, consistency between individual force measurements was difficult to accomplish.

### 6.3.7. Investigation of the influence of potential optical artefacts

The variations in force measurements were caused by several technical challenges. In Figure 6.12.A. one such challenge is illustrated. As the movable bead was brought closer to the stationary bead, the optical trap of the traced bead appeared to move towards the moving bead. This resulted in a force profile of the type shown in Figure 6.12.B, with minima and “negative” forces in the profile. These force troughs impeded the force measurements as the traced bead appeared to be propelled in the wrong direction (in this case to the right), which caused the force calculation software to interpret this movement as negative force.

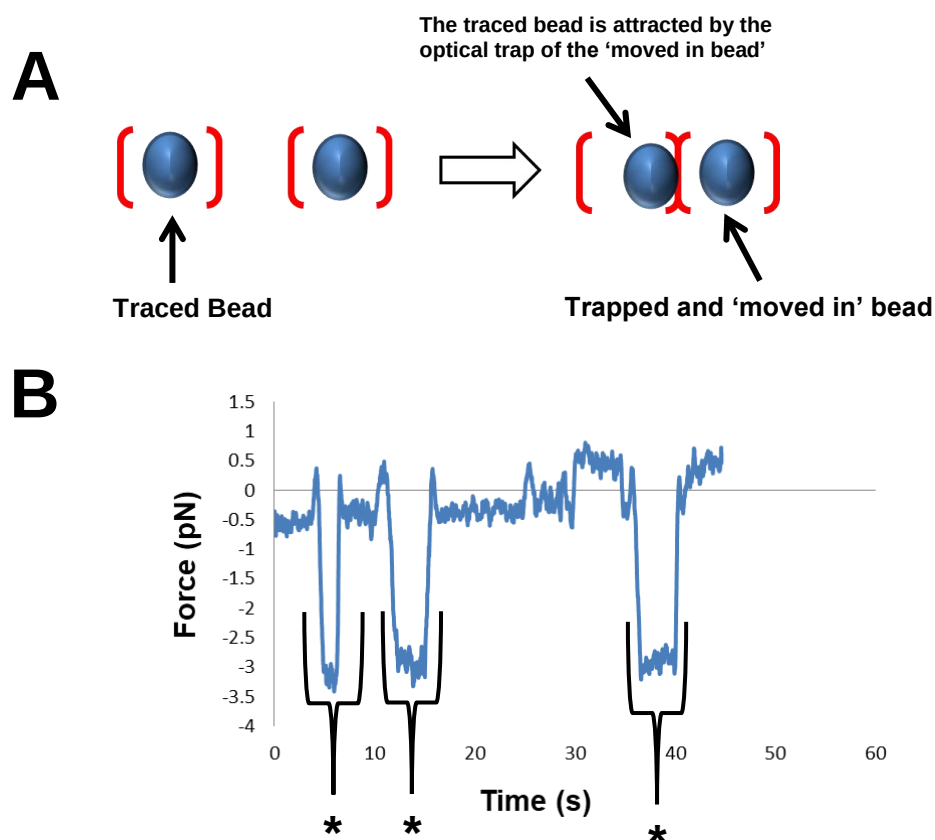


Figure 6.12. Measurement errors within the HOT set up. (A) As the trap on the left, moveable bead was moved close enough to the right, the trap of the moveable bead appeared to attract the traced bead on the right and interfered with the force measurement. (B) The phenomenon shown in (A) created negative force values (indicated by the asterisk marked troughs) and correct force measurements were no longer possible.

Because reproducibility in force measurements was difficult to maintain, potential additional reasons for the origins of the variations in the measurements were then investigated systematically. To this end, the first parameter investigated was the optical trap proximity. Here, experiments were carried out in which a single bead was tracked and traced using the HOT and a second trap, either empty (Figure 6.13.A) or holding another bead (Figure.6.13.B) was moved defined distance steps ( $1\text{ }\mu\text{m}$ ) closer to the tracked bead. It was found that the position of the stationary, traced trap used to measure forces on the stationary bead varied according to the position and proximity of the trap that was moved closer to it.

Figure 6.13.A and Figure 6.13.B respectively, show that as an empty trap or a trap holding a bead was brought closer to the tracked bead (held stationary), the X and Y recorded position of the bead started to vary in a sine wave like fashion. The experiments were repeated three times for three different beads. This phenomenon was most likely caused by interference between the optical trap holding the movable bead and the optical trap used to hold the traced bead stationary in the same position. Airy discs surrounding the points where the light hits the objective to create the traps, can produce regions of constructive or destructive interference and thus could result in the sine wave like periodic variations observed in Figure 6.13. This meant that in force measurements with HOT the distance between optical traps needed to be considered before force measurements could be made.

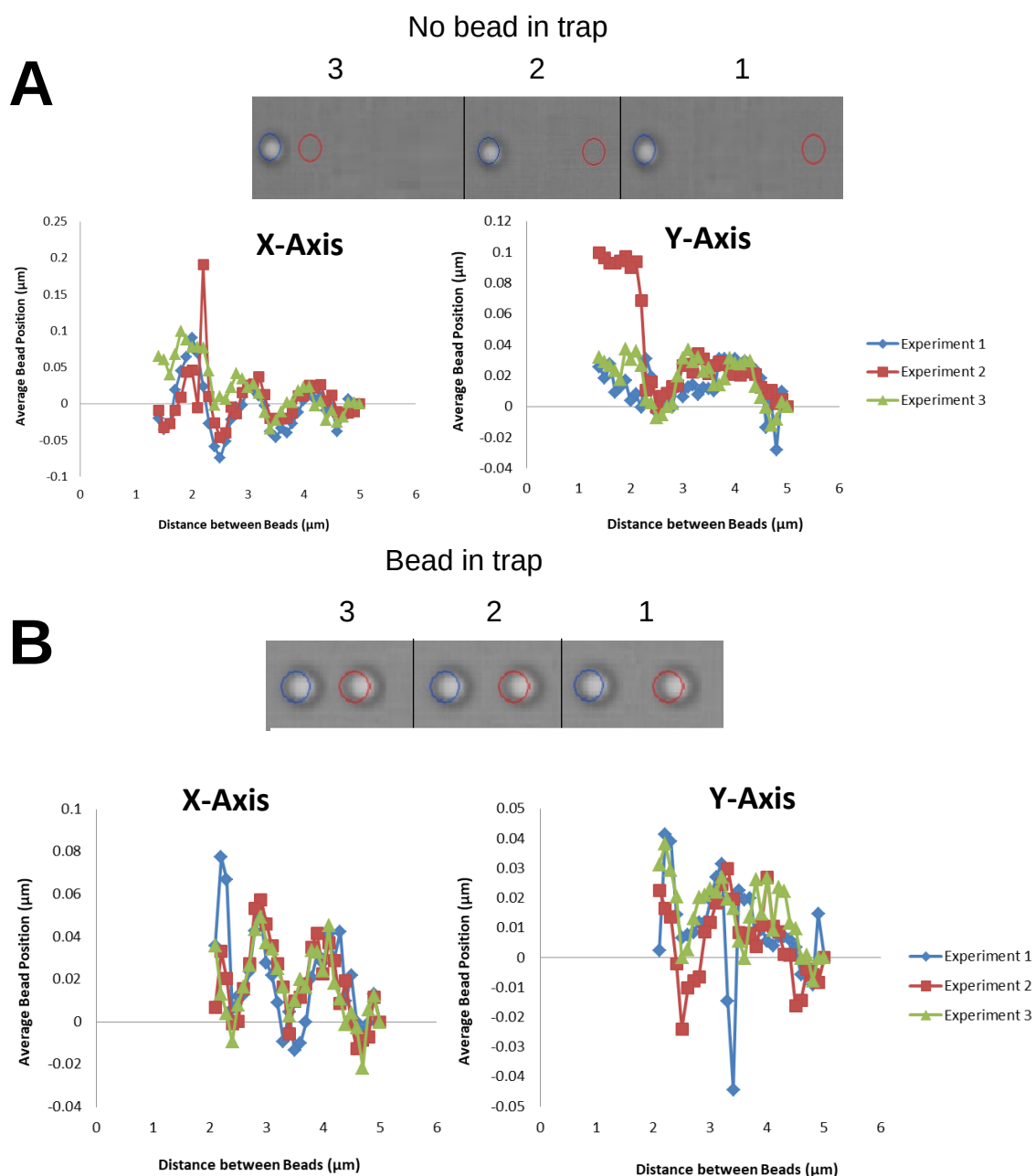


**Figure 6.13.** Proximity experiments to investigate the source of the erratic force measurements produced by the HOT set up. (A) The average position of a trapped, stationary and traced bead was measured as a second empty trap was moved closer in defined intervals of 1  $\mu\text{m}$ . The stationary bead position varied in its X and Y coordinates in a sine wave like fashion as the empty trap is moved closer. (B) The same trend was observed when a trap was moved closer that was holding a bead. Note that the beads were moved closer but not made to touch. A maximum distance of 2  $\mu\text{m}$  was kept between the beads. The experiments were repeated three times for three different beads.

The stiffness of the optical trap was also found to be dependent on the number of optical traps created with the HOT. Figure 6.14.A shows how two empty traps were formed and moved closer to a stationary, traced bead in defined intervals of 10  $\mu\text{m}$ . The experiment was repeated three times for three different beads. The trap stiffness of the traced optical trap decreased as the two traps were formed. This was due to the laser power of the laser beam being split to form multiple traps by the SLM and thereby reducing the available light intensity available for the stationary, traced trap (see Chapter II, Section 2.4.1.).

Although the formation of multiple optical traps affected trap stiffness, the proximity of those traps to the stationary, traced bead did not impact trap stiffness (Figure 6.14.B).

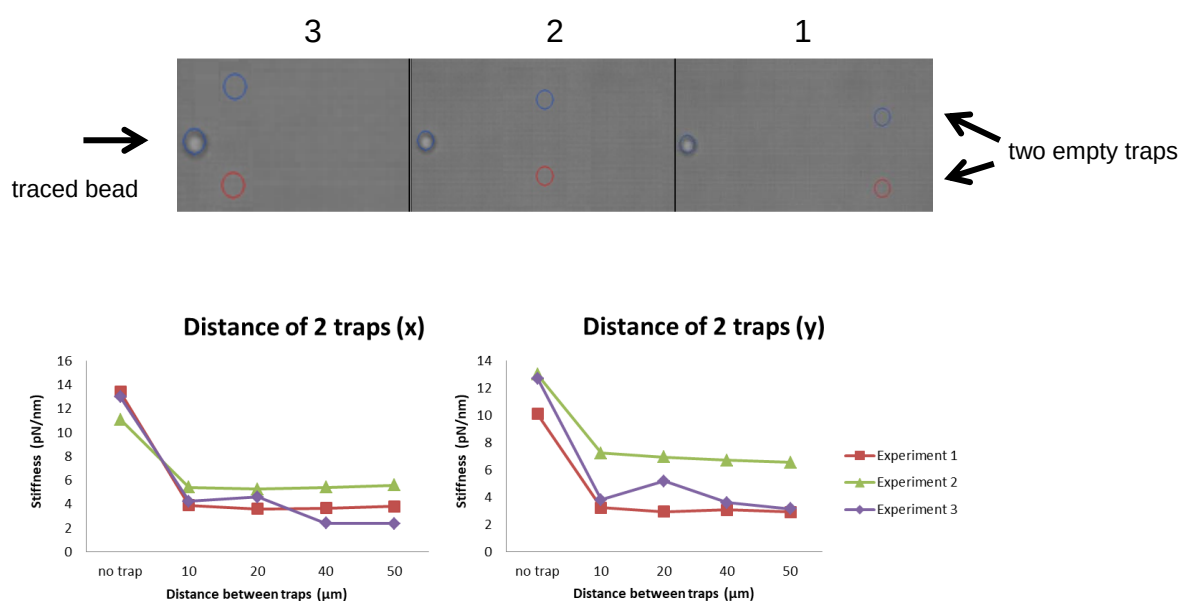


**Figure 6.14. Impact of multiple optical traps on trap stiffness. (A)**Three optical traps were created with the HOT. The trap on the right was used to trap a bead and measure trap stiffness and the two empty traps on the left were moved closer in defined intervals of 10  $\mu\text{m}$ . **(B)** When the two traps on the left were formed the trap stiffness of the single, right trap holding the bead was reduced but that trap had consistent stiffness values even as the two the traps on the left were moved closer to the right, stationary, traced bead.

### **6.3.8. Approaches to overcome HOT technical issues**

To avoid the production of erroneous force curves and overcome interference issues it was attempted to introduce larger distances between the traced and moved bead in order to e.g. to overcome the issue identified in Fig.6.13. One such attempt was to use a bead 'barrier' that can introduce more distance between the traced and the moved bead.

To this end two E-cadherin coated beads were brought into contact using the HOT and held in contact for at least five minutes. After holding the beads together for a sufficiently long time they stuck to stick together strongly due to the E-cadherin on the bead surface. A third E-cadherin coated bead was captured with the HOT and pressed to the meeting point between the two previously joined beads (see Figure 6.15.A). The two beads that were joined initially formed a barrier to introduce space between the traced and movable bead during force measurements.

Although some improvements in the quality were observed e.g. measurements with force profiles similar to those shown in Figure 6.10.B. could be produced using this technique, it was not possible to significantly reduce the variance between individual measurements. This disqualified this approach as a solution of the trap interference issue.

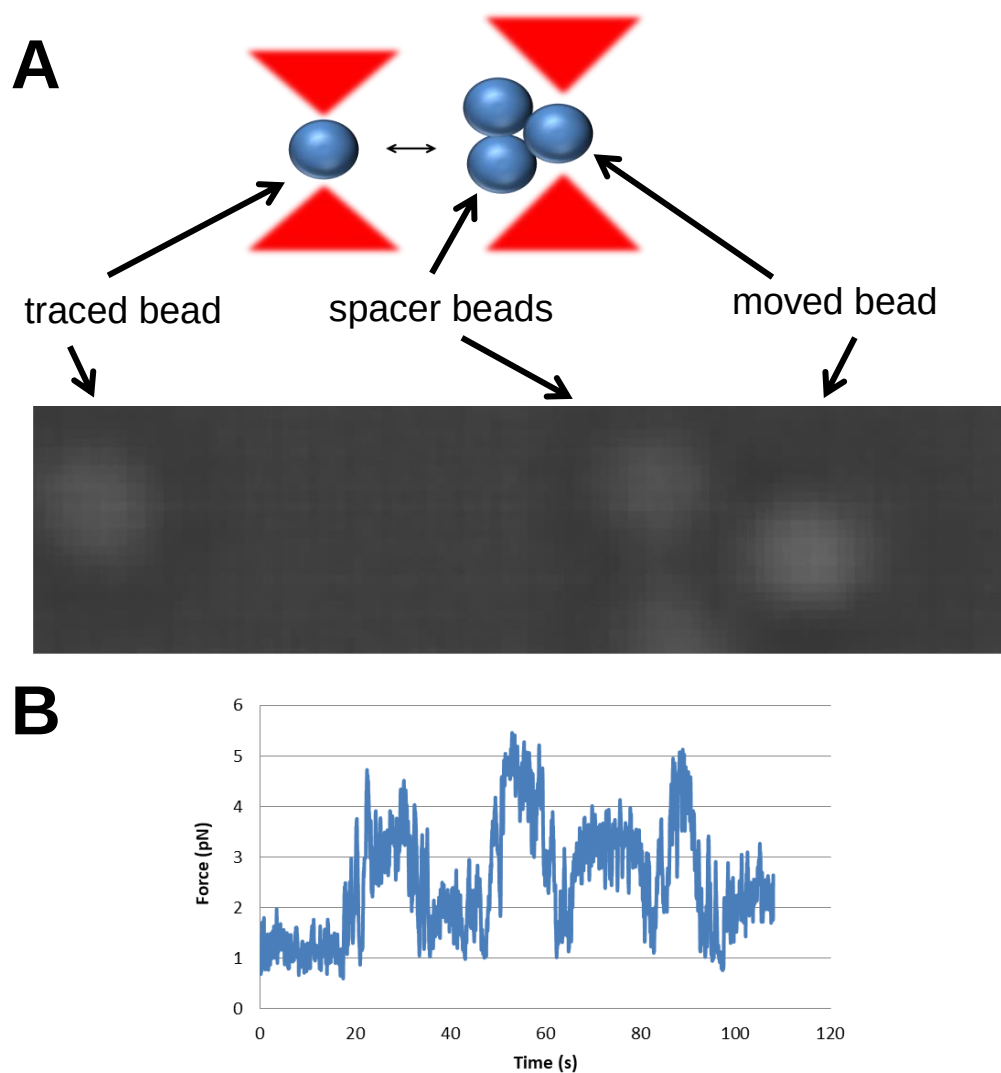


**Figure 6.15.** Bead barrier experiment to correct erroneous force curves produced by the HOT set up. (A) A barrier is formed by holding three E-cadherin coated beads together for at least five minutes using the HOT (see main text). This created a barrier intended to create enough space to overcome interference of two traps. (B) Although it was possible to measure interaction forces with this set up, consistency between individual force measurements as well as high rates of successful measurements was difficult to accomplish.

Another attempt to increase the distance between stationary and movable beads was therefore the use of more defined 3D printed polymer ‘spacer’ micro structures printed in poly(lactic acid) that could be easily trapped by the HOT. Star (Fig. 6.16.A) and dumbbell (Fig. 6.16.B) like structures were designed with the CAD parametric 3D modeller and printed using the Nanoscribe Photonic Professional GT 3D printer in collaboration with Mr.Jacob Mazza (Lehigh University, USA) and the Institute for Advanced

Manufacturing (University of Nottingham). As can be seen in Figure 6.16.B the original sphere design of the silica beads with a diameter of 2  $\mu\text{m}$  was retained. The bead design was integrated into the star or dumbbell structure.

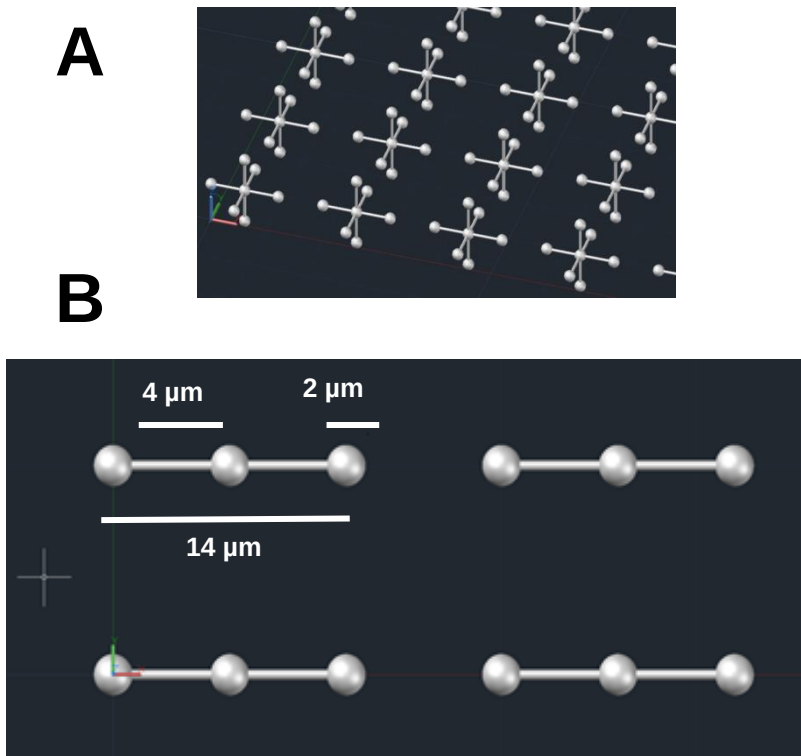


**Figure 6.16. 3D printing of microstructures to provide spacer structures. The original sphere design of the silica beads with a diameter of 2  $\mu\text{m}$  was retained. (A) Star like design for microstructure for the spacer structure in CAD software interface. (B) Dumbbell design for the spacer structure in CAD software interface.**

Both structures were designed so that the central bead could be captured with the HOT and the external part of the construct then be used to move the structure into contact with the traced bead while introducing enough distance between the optical traps so they would not overlap. The structures could be successfully printed and tested with the HOT. Figure 6.17. shows the appearance of 3D printed structures of the dumbbell design under the microscope of the HOT on their printing base platelets.

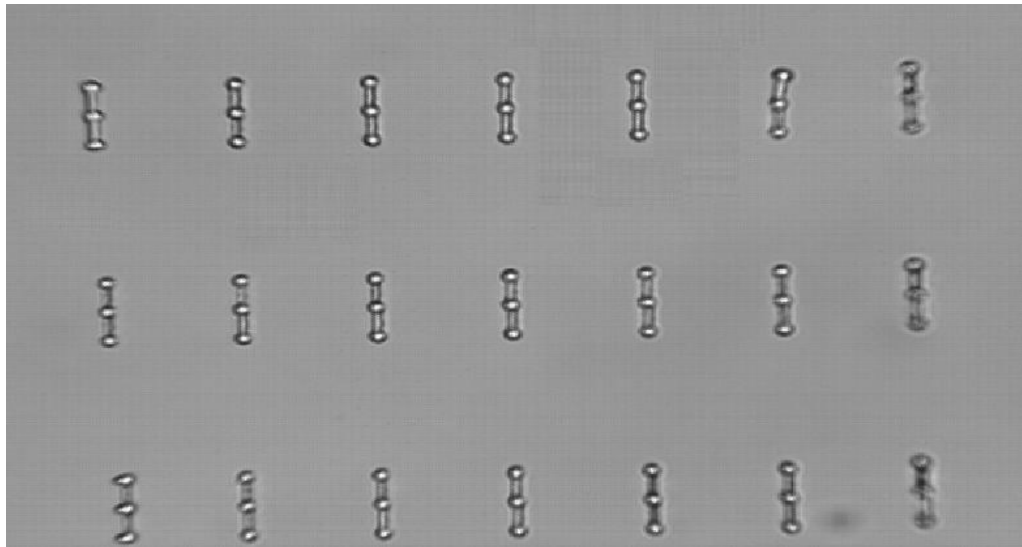


**Figure 6.17. 3D printed structures of the dumbbell design under the microscope of the HOT on their printing base platelets. Only the dumbbell design of the structures could be successfully printed and tested with the HOT.**

However, as only small quantities of 3D printed structures could be produced at one time it was difficult to locate and handle the construct samples in the comparably large liquid volumes required for HOT experiments. Furthermore, because the HOT had to be used to dislodge the structures from their printing base platelets scenarios could occur in which it was impossible to get the structures into solution as the forces created by the HOT were not strong enough get the constructs off the printing base. Due to obstacles such as these, this approach could not be developed to its full potential within the time confines of this project.

### **6.3.9. Measurement of mES cell interactions using HOT**

Despite the difficulties in measuring forces using the HOT, we attempted to use the approach described in section 6.2.4 to measure the E-cadherin interactions on the surface of mES cells. The force measurement procedure described in section 6.3.5. was adjusted to suit the requirements of working with mES cells. Cells suspended in solution could be captured with the optical trap and freely moved in all 3 dimensions. To avoid unwanted adhering of the cells to the slide bottom and the cover slide the glass had to be pre-incubated for at least 15 minutes in 5% polyhema (poly(2-hydroxyethyl methacrylate)(Sigma)). mES cells were reconstituted in a concentration of 2500 cells/mL in mES cell culture medium, mixed with silica beads (with the same bead concentration as in the viscosity and force measurement experiments, see Section 6.2.3. above), loaded onto a glass slide and sealed by gluing the cover glass slide on top of the sample area. The concentrations for mES cells and beads were again empirically determined as part of the original work of this thesis and again iterative investigation was necessary to determine the right ratios of the afore mentioned materials.

These experiments showed that it was possible to trap beads and mES cells at the same time and bring them into and out of contact using the HOT. Figure 6.18.A shows a trapped bead and a mES cell in suspension under the HOT microscope. Cells could be held using two optical traps to hold the upper and lower part of the cell. To make force measurements a bead was trapped, kept stationary and its movements again monitored. A mES cell was captured and held with two traps. Now the cell was moved towards and away from the trapped bead to induce adhesion between the bead and the cell.

However, although it was possible to move single mES cells in this way it was very difficult to record force measurements with the HOT due to the measurements being diminished by the physical size of the cells: e.g. when in close proximity, the camera was not able to discriminate between the bead and the cell. Figure 6.18.B shows a series of experiments to illustrate the difficulties of working with HOT on mES cells.

A cell was moved closer to the stationary bead in steps of 10  $\mu\text{m}$ . The stiffness of the trap holding the bead started to change as the cell was moved closer and high quality force measurements were no longer possible, similar to the trap proximity experiments described in Section 6.3.7. This experiment was repeated three times for three different mES cells and beads respectively. The observed trend could be attributed to the fact that the stiffness of the trap holding the stationary bead decreased as multiple traps are formed using the SLM. As the cell was moved closer, the stiffness of the trap holding the bead was not able to hold the bead stable in the same position anymore. Thus fluctuations in bead position resulting from a decline in trap stiffness did not permit accurate force measurements with HOT to be made.

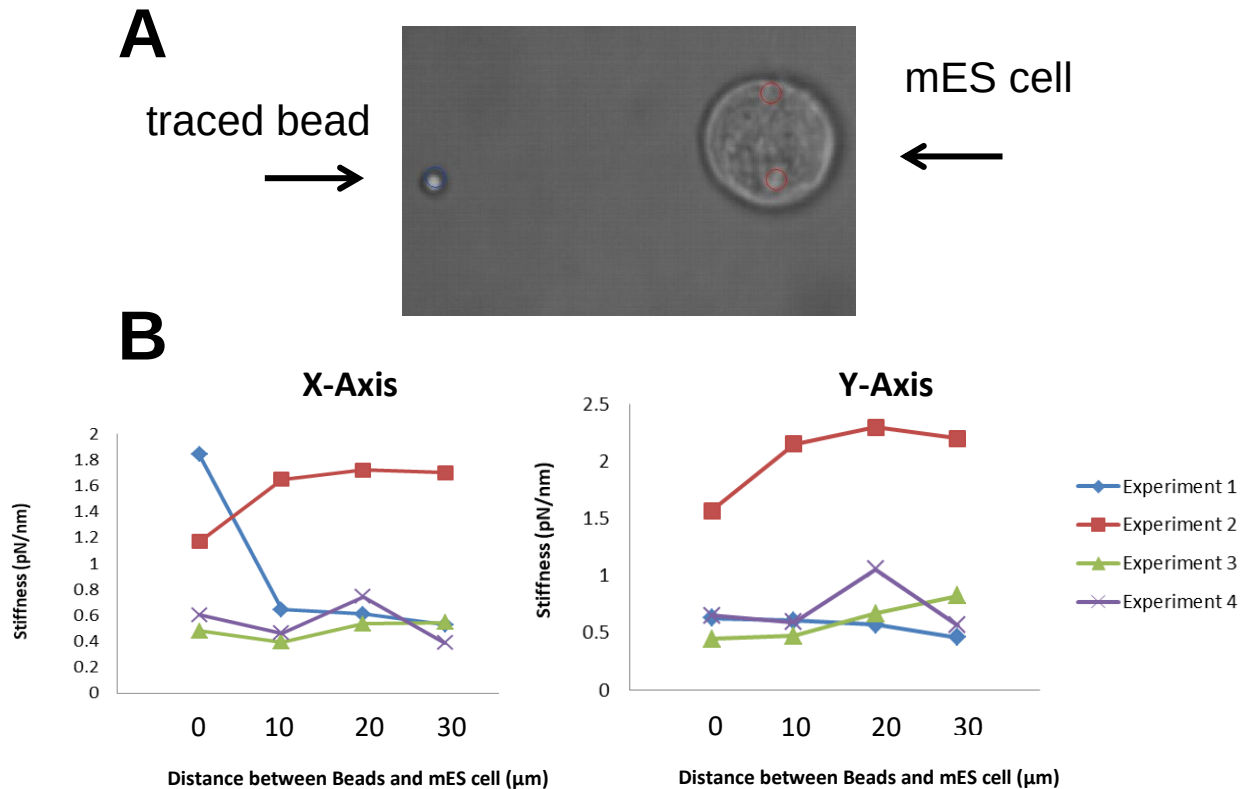


Figure 6.18. Proximity experiments to investigate the source of the erratic force measurements produced by the HOT set up in force measurements on mES cells. (A) To make force measurements a bead was trapped, kept stationary and its position was monitored (on the right). A mES cell was captured and held with two traps (on the left). Now the cell was moved towards and away from the traced bead to induce adhesion between the bead and the cell. (B) As the cell was moved closer to the stationary bead, the optical trap started to vary in its stiffness. The experiment was repeated three times for three different mES cells and beads respectively. These fluctuations were likely to be caused by a decline in trap stiffness as a result of multiple optical traps being formed with the HOT. The fluctuation of traced bead position due to changing trap stiffness did not permit accurate force measurements to be made with the HOT.

## 6.4. Conclusions

A HOT set up was successfully used to demonstrate viscosity and force measurements. This was made possible by investigating the dependence of the optical trap on various factors such as sample viscosity, applied laser power, number of optical traps formed, proximity of optical traps and position of the optical trap in the focal plane. HOT could measure the viscosities of media precisely and accurately as already reported in the literature. The HOT

set up was found to reach its detection limit for glycerol viscosities after 50% of glycerol, which can be attributed to a match in refractive index of bead and solution.

It was possible to use HOT to measure the interaction forces between uncoated silica beads or beads that were coated with E-cadherin. Beads and mES cells could be trapped at the same time and they could be brought into and out of contact using the HOT. Variations in force measurements were investigated and found to be caused by several technical challenges such as the overlapping of optical traps and camera resolution limits. To overcome interference issues and to avoid the production of erroneous force curves it was attempted to introduce larger distances between the traced and moved bead by using bead barriers and 3D printed micro-structures. Although some improvements in the measurement quality were observed using these techniques, it was not possible to significantly reduce the variance between individual measurements within the time confines of this project.

Despite problems with reproducibility and comparability of these measurements with AFM experiments, the HOT were here, for the first time, successfully employed to study E-cadherin mediated interactions on the surface of mES cells.

# **Chapter VII**

## **Conclusions and future work**

E-cadherin is a useful target to study mechanical signals in embryonic stem cell differentiation. It is associated with the maintenance of pluripotency and it functions as an adhesive molecule. This research project aimed to explore cadherin interactions on the surface of undifferentiated embryonic stem cells with atomic force microscopy (AFM). Furthermore it was attempted to examine how these interactions change as ESC's differentiate into particular lineages particularly, the neuronal lineage.

To accomplish these objectives, it was demonstrated in Chapter III, that functional cadherin domains could be immobilized on silicon surfaces and AFM probe tips. Bond formations of recombinant E- and N-cadherin ectodomains attached to tips and surfaces could be induced and the force necessary to break these bonds quantitatively measured. The obtained force values ranged from 20-80 pN and were in agreement with those previously reported in literature <sup>12, 15, 117, 118</sup>. Observed interaction forces were predominantly attributed to single molecule events based on their binding frequencies <sup>85, 86</sup>. The binding frequency could be substantially reduced by exposure to known cadherin binding inhibitors such as EDTA, EGTA and cadherin specific antibodies which also demonstrated the specific nature of cadherin bond formations.

In Chapter IV, a procedure was developed to derive neuronal precursor cells from undifferentiated mES cells. The presence of E- as well as N-cadherin on the surface of undifferentiated mES cells and progenitor cells at different time points along the neuronal differentiation pathway was demonstrated using immunohistochemistry (IHC). By labeling E- and N-cadherin on the cell surface as well as the DNA inside the nucleus it was possible to compare the fluorescence intensity of cadherin relative to the DNA stain (Hoechst), and it was found that the fluorescence intensity for E- cadherin underwent a marked decline through the progression of differentiation stages towards neuronal precursors. N-cadherin levels did not display such trends and only increased to a small degree in the neuronal precursors. After having demonstrated the presence and change in expression level of E- and N-cadherin on the surface of mES cells and differentiated neuronal precursor cells it was then possible to apply the insights gained in Chapter III about the study of cadherin interactions at the single molecule level using AFM, to the study of cadherin interactions on these cell types.

In Chapter V, cadherin interactions between E- or N-cadherin cadherin constructs on AFM tips with native cadherin expressed on mES cells or neuronal precursors were examined using AFM. It was possible to demonstrate that the recombinant constructs immobilized on AFM tips were able to form molecular bonds with native cadherin on the cell surface. The Interactions could also be blocked through administration of an antibody specific for the cadherin type on the AFM tip, which provided proof of the authenticity as well as the specificity of

the molecular bonds investigated with AFM. No substantial differences could be observed between the E- cadherin or N-cadherin interactions recorded on functionalized surfaces or on mES cells in response to externally applied force or in their binding frequency. For example, in both SMFS and in CFS regardless of the cell type, E- and N-cadherin bond rupture forces were found to decline with increasing AFM tip speeds. Strong similarities could also be observed when measuring the rupture forces of E- and N-cadherin bonds in SMFS experiments and on the surface of undifferentiated mES cells at different tip speeds (i.e. <10 pN difference).

Preliminary experiments highlighted potential differences in experiments when mES cells were allowed to differentiate into precursor cells of the neuronal lineage. E-cadherin bond strengths were found to be higher when probed on the surface of NP cells. Although there is limited data to support the findings on neuronal precursor cells, there were indications that a lineage specification of mES cells may go hand in hand with a change of E-cadherin mediated adhesion. These changes are realized not only through changes in cadherin expression levels but possibly also in E-cadherin bond strength.

Finally, in Chapter VI, as an alternative approach to AFM, holographic optical tweezers were utilized to investigate cadherin interactions on mES cells. HOT possess a unique potential to explore the nature of cadherin interactions, with a greater resolution of detecting bond rupture forces compared to AFM and have the ability to probe interactions directly between two cells.

It was possible to measure interaction forces between trapped silica beads using HOT. Forces could also be investigated between E-cadherin coated beads and mES cells. However the close proximity of optical traps which occurs during such measurements was found to cause interference and erroneous force curves, including sine wave like deviations in the traced bead position as one trap closed in on the other. Difficulties such as these prevented the work from progressing within the timescale of this project. As potential solutions for the interference issues on the HOT bead and 3D printed micro structures were developed and tested that were intended to provide enough distance between the traced and the moved bead to prevent trap interference.

The main finding of this thesis was that the interactions of E-cadherin could be explored for first time on the surface of undifferentiated embryonic stem cells using AFM. Although this approach is still in the early stages of development, the results described here highlight the sensitivity and potential of AFM studies assessing cadherin interactions to study mES cell behavior.

In future experiments, the differentiation state of the embryonic stem cells studied will have to be more rigorously verified using defined marker genes and proteins such as as SSEA (1,3,4) <sup>40</sup>, E-cadherin <sup>46-55</sup> and CD 9 <sup>157</sup> for pluripotent mES cells and NeuN <sup>158</sup>, MAP-2 <sup>159</sup>, and NSE <sup>160</sup> for terminally differentiated none mitotic neurons. For example, to achieve this one would apply e.g. high precision sequencing techniques such as quantitative polymerase chain reaction (qPCR), Western or Northern blot, microarray analysis and fluorescence in situ hybridization (FISH).

Furthermore in order to test to what extent the recombinant cadherin ectodomain used for the experiments of this thesis differs from native cadherin, an alternative set up for force measurements on cells could be developed. This approach could involve a cell that is attached to a cantilever of an atomic force microscope. By combining atomic force microscopy (AFM) and optical microscopy, cells could be positioned to induce cadherin interactions at a given location, on another cell, on a functionalized surface or on a tissue sample. In this way the interactions of native cadherin on the surface of mES cells would be investigated rather than that of a recombinant, possibly compromised fusion construct.

Another alternative to advance this research approach would be to test silicon surfaces functionalized with different surface densities of cadherin and with truncated versions of the ectodomains (e.g. ectodomains that only have the EC1 to EC2 part of the protein). This would enable the assessment of the impact that variations in these subunits have on cadherin mediated adhesion of embryonic stem cells, thus revealing more information about the active binding mechanism of the cadherin molecule itself.

With further development this approach could be used as a tool to advance stem cell research by identifying the pluripotent state as well as other stages of development based on physical rather than by using chemical or genetic cues. This could be of great benefit in cancer research when discriminating between normal and cancerous cells using AFM, as well as in regenerative medicine, tissue engineering, drug discovery, disease modelling and developmental biology. The approach could also be employed to investigate the basis of agents

which may modify cadherin interactions. Indeed this is currently being explored in projects within the research lab

# **Chapter VIII**

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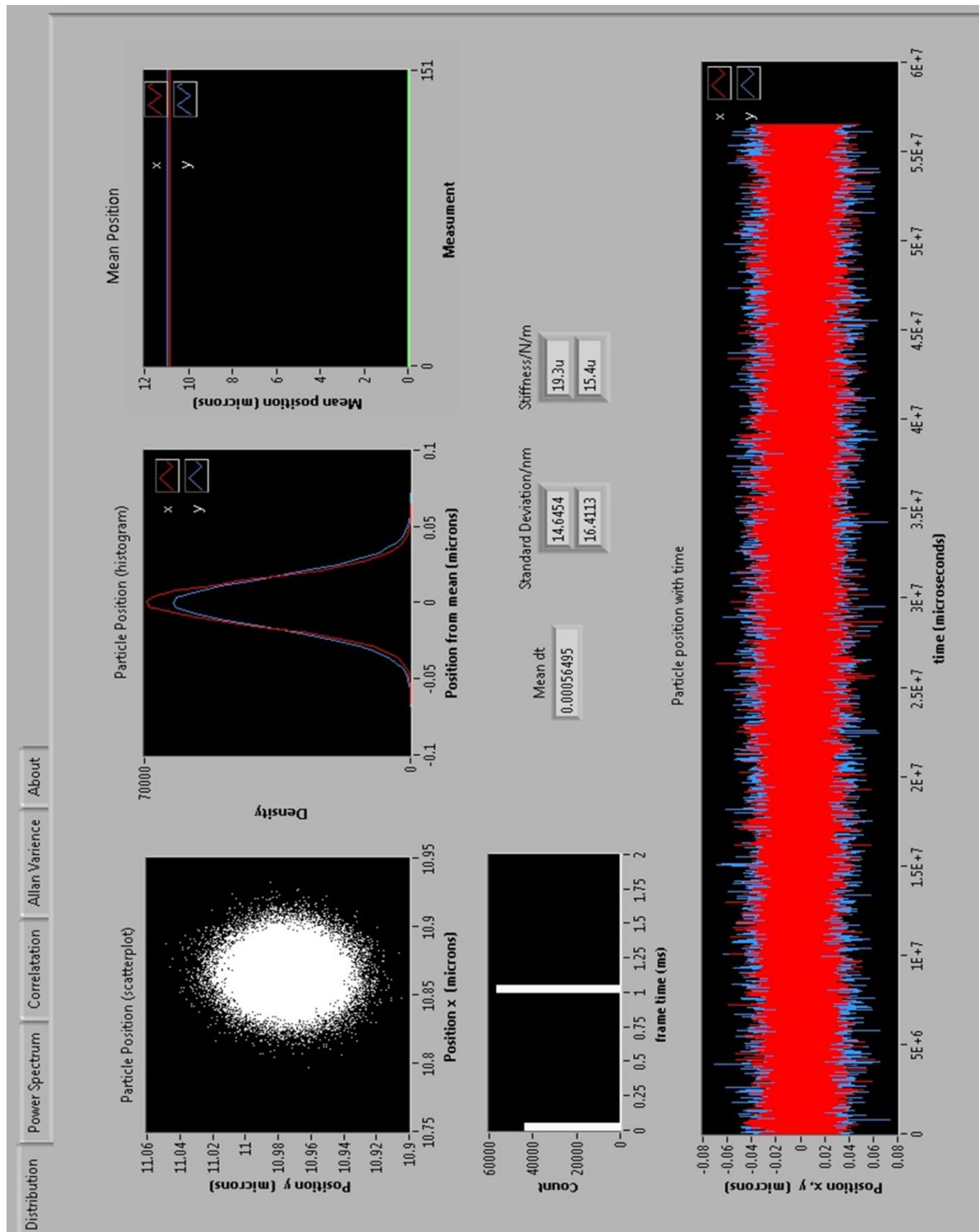
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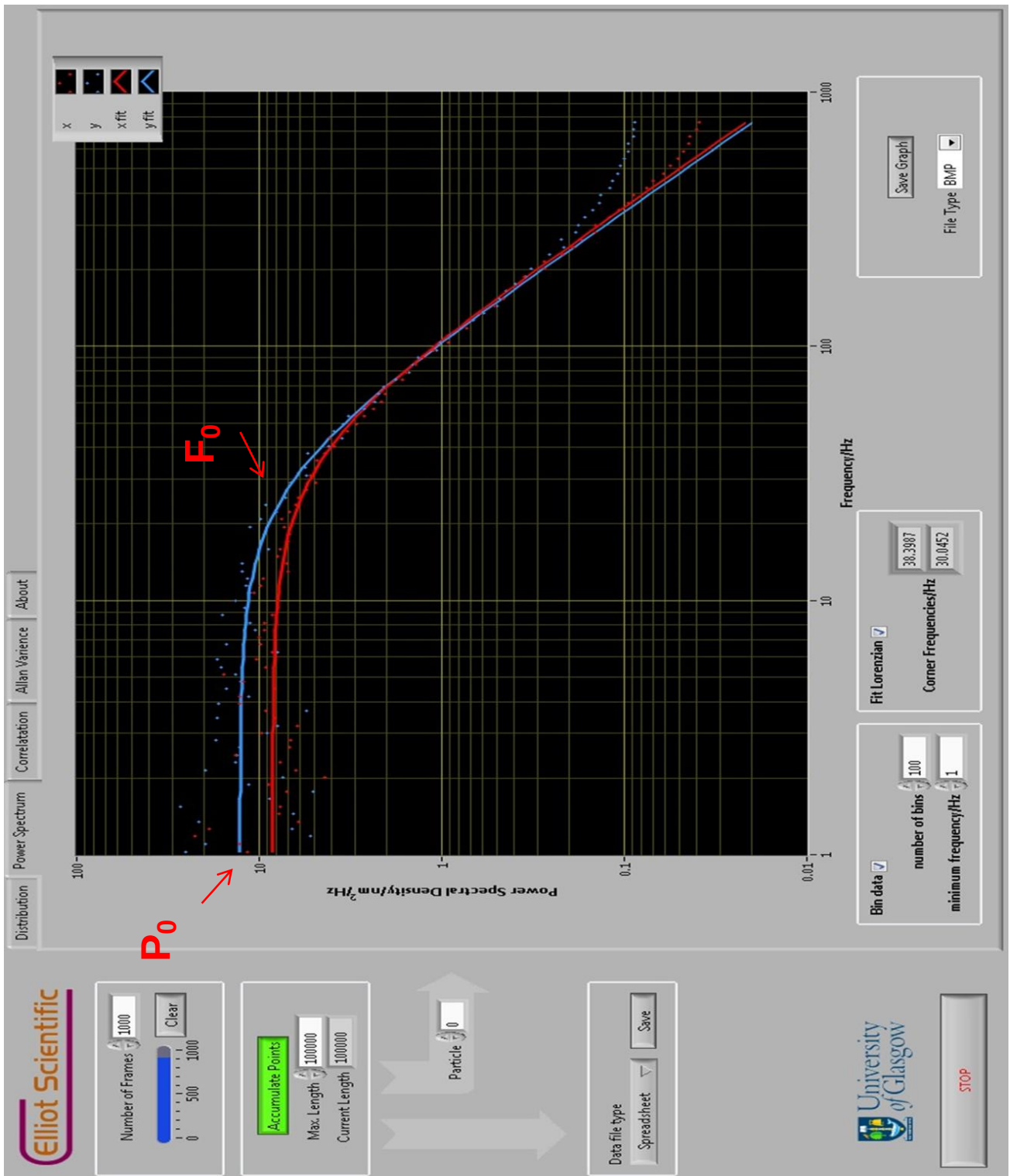
# Appendix

## A



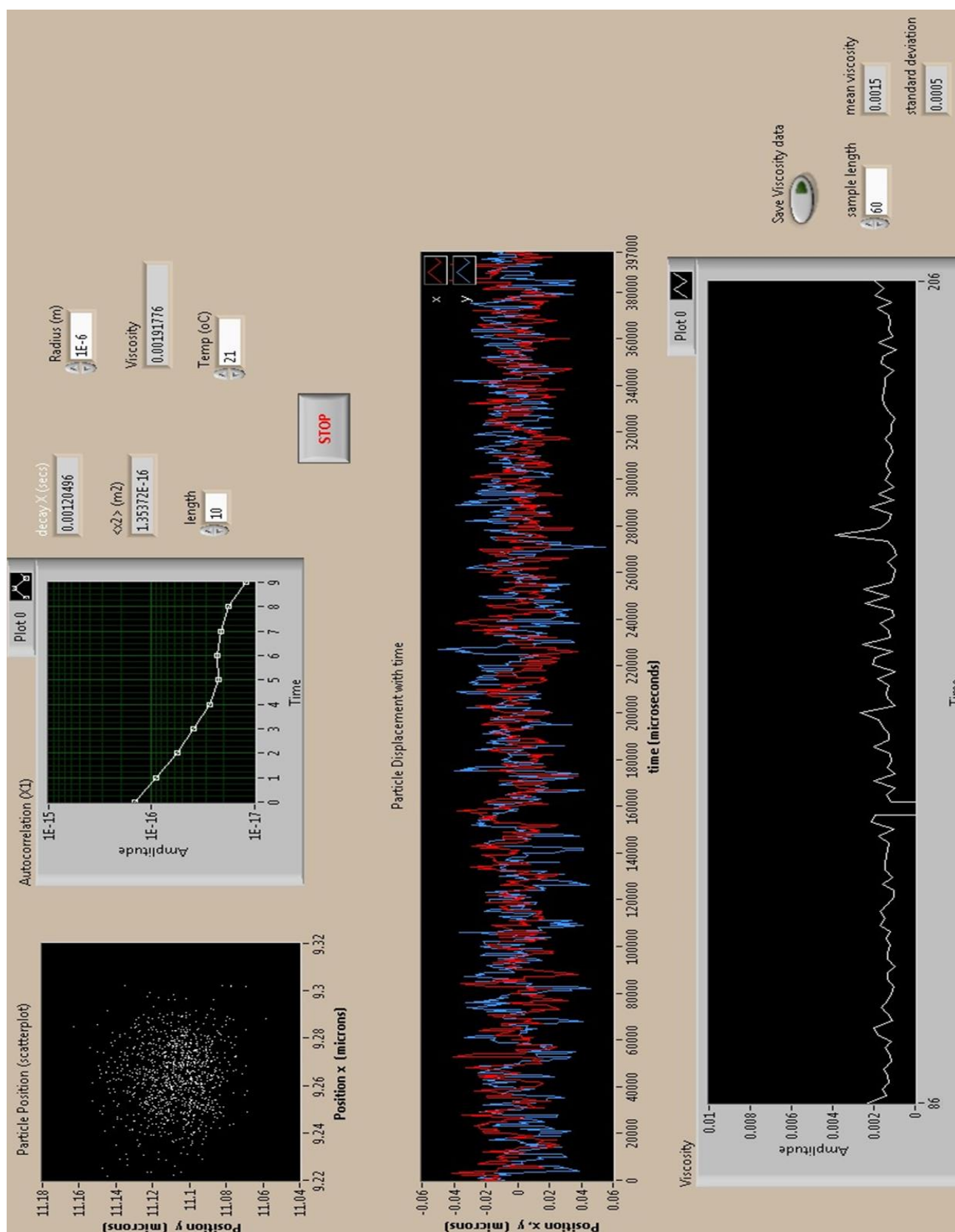
Appendix (A) Software interface of trap stiffness measurements. The trap stiffness was measured by recording the mean squared displacement of the bead  $\langle x^2 \rangle$ .

**B**



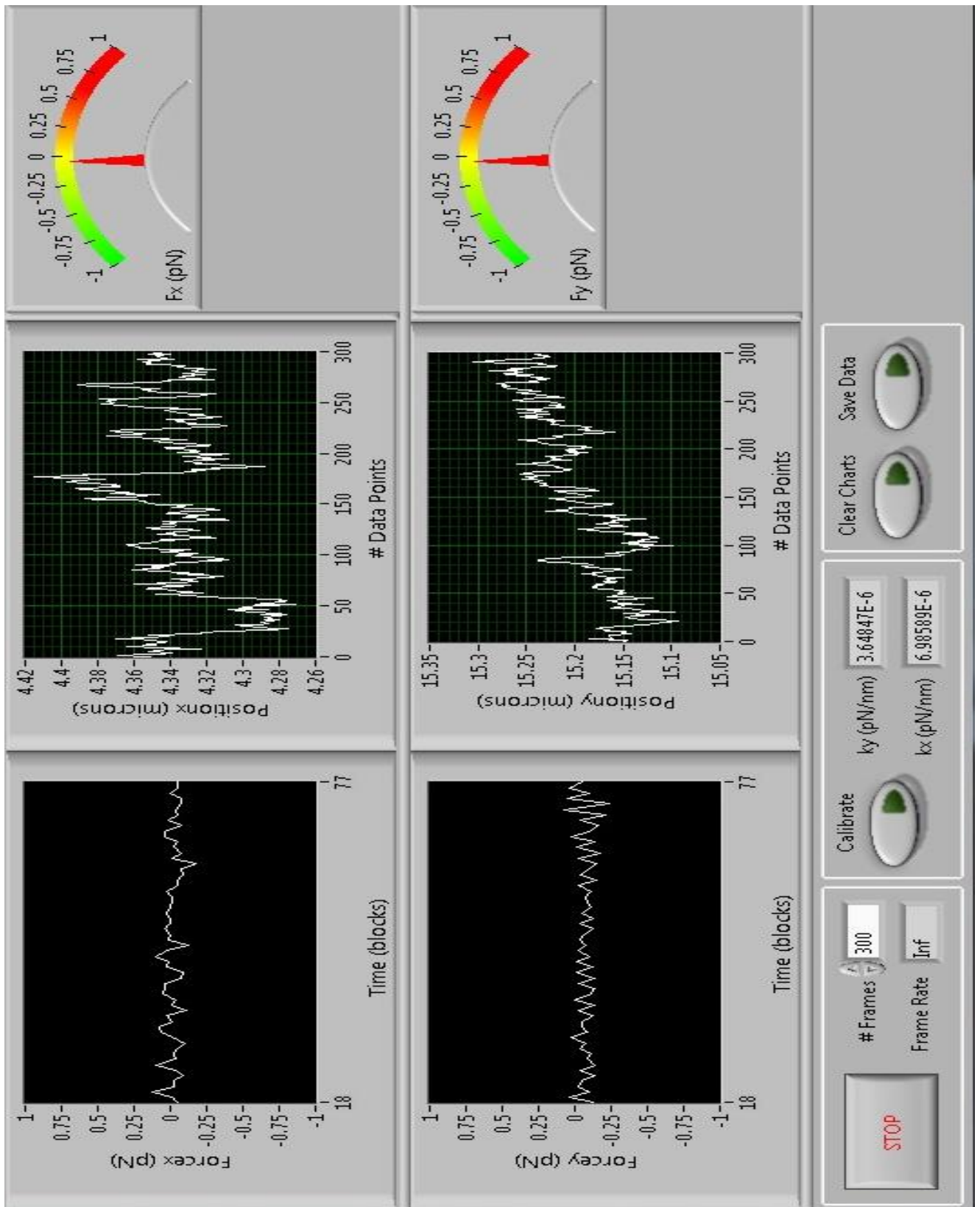
Appendix (B) Software interface of the power spectrum. By using the curve fitting software the low frequency intercept  $P_0$  and the corner frequency  $F_0$  are estimated. The red and the blue line represent the best fit of the model to the data for particle movement in X (blue) and Y (red) direction.

C



**Appendix (C) Software interface of viscosity measurements. With the mean squared displacement of the bead  $\langle x^2 \rangle$  and the characteristic decay time of the system  $\tau$ , the viscosity of the solution could be measured.**

**D**



**Appendix (D) Software interface of force measurements. The change in position of the bead inside the trap was converted using Hooke's law to get the force in X and Y plane of the optical trap necessary to keep the bead inside the trap.**