Appendix II

Appendix I describes how the data is derived on the flow cytometer to allow an estimation of the proportion of cells in G_1/G_0, S and G_2/M (the viable fraction). Shown here is an example of data extraction and how the final histograms are derived.

The data was analysed using the available software packages; Expo32™, WinMDI and Cylchred. Each set of data was analysed individually as described in Appendices I and II and the data pooled together to obtain the average proportion of cells in the viable fraction for each time point and condition. In pooling the data, an estimate of the SD was also obtained, allowing us therefore to have an average±SD for each time point and condition and it is this data which is depicted in figures 3.6, 3.8, 3.10, 3.12, 5.3 and 5.8. It is also this data that was applied to the mathematical model as outlined in chapter 5. In addition to calculating the proportion of cells in G_1/G_0, S and G_2/M, an estimate of the proportion of cells in the pre-G_1 compartment was also calculated. There are several methods by which this can be done and are described in section 3.3.

Figures 1 and 2 aim to coherently describe how the data was extracted and analysed.

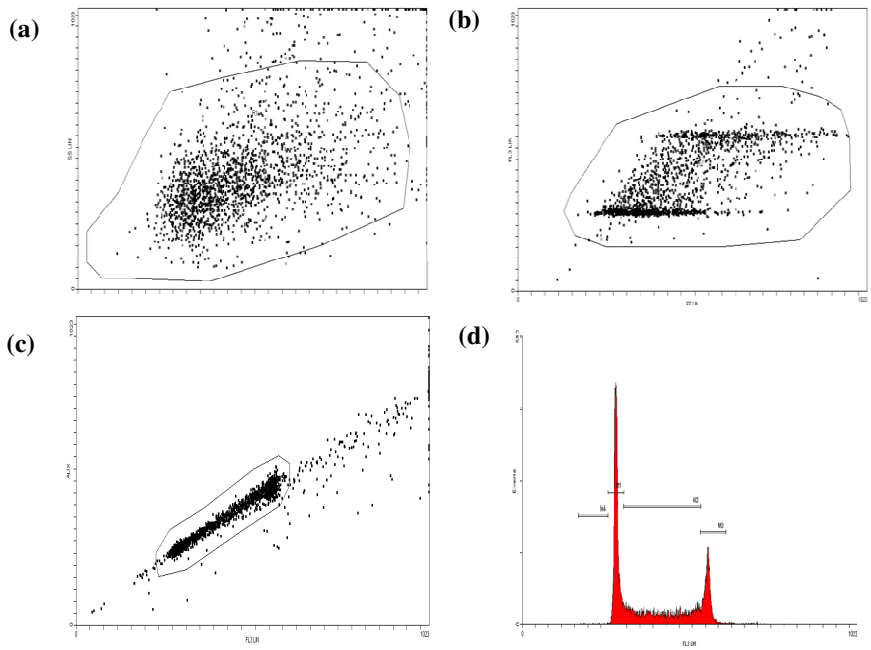


Figure 1: Data Extraction from control KM12 cells. The dot plots in (a), (b) and (c) depict the gating used to define the final histogram in (d). Dot plot (a) allows us to gain an estimate of the viable fraction of cells Dot plots (b) and (c) are both gated using dot plot (a) and allows us to define the single cells within the population of cells therefore ensuring the histogram in (d) is a picture of the cell cycle of the single cells only.

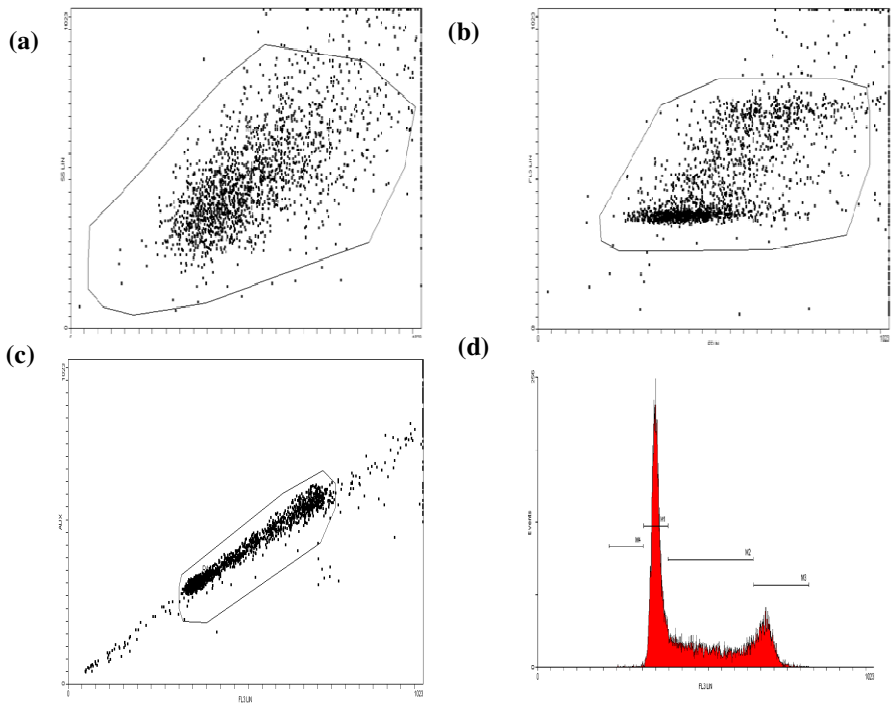


Figure 2: Data Extraction from KM12 cells incubated with 100 µM RHPS4. Cells incubated with varying concentrations of RHPS4 were analysed in the same way as control cells. The dot plots in (a), (b) and (c) depict the gating used to define the final histogram in (d). Dot plot (a) allows us to gain an estimate of the viable fraction of cells Dot plots (b) and (c) are both gated using dot plot (a) and allows us to define the single cells within the population of cells therefore ensuring the histogram in (d) is a picture of the cell cycle of the single cells only.

There are several ways of analysing the histograms shown in figure 1:

1. Manual segmentation of the histogram is achieved by placing markers.
2. Using the raw plot data of FL3 versus FSC-H would give the regions of the viable fraction. These could then be used to give sub-fraction distributions of the cell cycle.
3. A fitting algorithm can be applied, such as WinMDI or Cylchred.

The data shown in figures 1 and 2 are examples of the manual method and the analysis of the histograms via the manual method is sufficient to determine the short term effects of RHPS4, as shown in chapter 3, and was analysed using the Expo32™ software. In the present study, however, the primary aim is the development of a mathematical model of the cell cycle to determine the effects of RHPS4 and the major novelty is the long term nature of the data. At present, there is unfortunately no method or algorithm that is consistently reliable in the fitting of

all distributions and so the manual method is not sufficient, therefore, for the analysis of the long term data that will be applied to the mathematical model. As this is the case, for the long term data it is necessary to use the software packages, WinMDI (freeware provided by Joe Trotter of the Scripps Institute, La Jolla, CA) and Cyclhred (available from CytonetUK). WinMDI allows the histogram to be gated using the dot plots as decribed in section 2.2.7.2 in order to derive a 'clean' histogram which is then saved in a FCS Histogram. file format. Cyclhred is a software package with the ability to 'read' a file of the FCS Histogram. file format. Cyclhred allows an estimated of the proportion of cells in the viable fraction based on algorithms by Watson *et al*, 1987.

In addition to the viable fraction, the mathematical model requires an estimate of cell death. The method used in the present study for cell cycle analysis used PI, and was derived from Nicoletti *et al*, 1995. In using this method, a pre-G_1 fraction is identified. This pre-G_1 fraction, in addition to representing apoptotic cells is also representative of mechanically damaged cells as well as cells with reduced chromosome number. As this is the case, there is often an over-estimation of the proportion of cell death and consequently has an impact on the noise and analysis of the viable fraction and lead to an under-estimation of their proportions. The most accurate and robust way of analysing the cell death fraction would be the Annxin V or terminal deoxynucleotidyl transferase (TdT). Since the present study aims to use a systems biology approach to the analysis of RHPS4, a review of the different methods used to accurately determine the pre-G_1 fraction are presented in section 3.3.