Semi-automated cell counting in phase contrast images of epithelial monolayers

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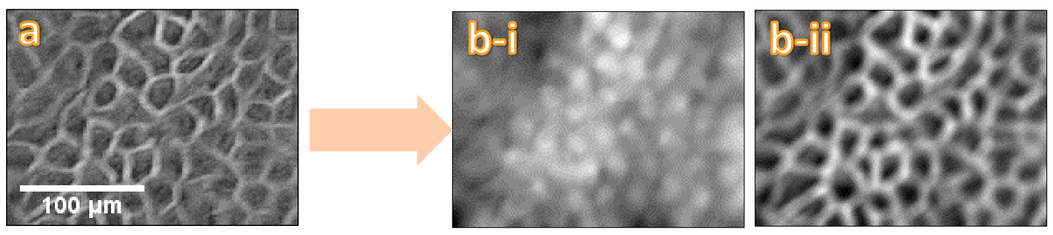
Introduction

- Keratinocytes cultured *in vitro* form monolayers, often used as a simplified, 2D model of epithelium to study cell behaviour. The growth of such cultures in response to a stimulus may be assessed using cell counts obtained at multiple time-points.
- Cell counting using a haemocytometer is destructive so it is necessary to simultaneously maintain several cultures to generate growth curves, which requires both time and resources. Furthermore, there is a high level of operator variability associated with this method [1].
- Phase contrast (PC) microscopy creates contrast non-invasively in optically transparent objects but images are subject to intrinsic artefacts in the form of the "halo effect", which cause cell segmentation to become non-trivial.
- Brightfield microscopy generally delivers poor contrast for unstained cells, but cells become brighter than background when the objective lens is moved slightly above the focal plane. The opposite contrast is observed when the objective lens is below the focal plane.
- Cells may be located and counted by subtracting two such images acquired with the objective lens displaced by 15µm [2], but this relatively small distance is difficult to achieve consistently without costly automated imaging apparatus.

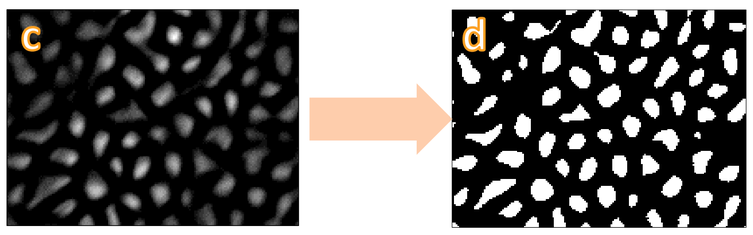
Image Analysis

Cell segmentation

- The contrast changes of defocusing brightfield microscopy can be emulated by applying a mean filter with a circular kernel to a single, in-focus PC image (image a)
- When the kernel radius , r_k , is larger than that of a cell, cells retain bright edges from the halo and a relatively dark centre (image b-i)
- When r_k is smaller than that of a cell, cells retain bright edges from the halo and a relatively dark centre (image b-ii)



- Imposing a minimum pixel intensity of 0 and subtracting the small r_k image from the large r_k image generates an image in which cell containing regions are brightest (image c)
- A single threshold may then be applied using the Otsu method to give an image in which cells are represented as binary objects (image d). The number of binary objects in an image gives the cell count.



- To select appropriate values of r_k , a ground truth dataset was obtained by manually counting the cells in 10 images representing a range of cell densities. A parameter sweep was performed to find the combination of large and small kernels that minimised the differences with the manual counts
- The average count from 20 images acquired at random locations in a culture was used to extrapolate total number of cells

Removal of erroneous segmentations

- Image noise and edge effects at low cell densities can cause incorrectly segmented regions, which contribute erroneously to the cell count and so must be discounted
- Morphological features of binary objects were used to classify each as either "cell" or "noise" using k-means clustering as detailed in figure 1.
- This analysis was applied to all segmented regions in all images acquired from a culture over the course of the experiment
- k-means classification success was assessed by comparison with 20 images across all samples in which all segmentations had been labelled manually, giving an F_1-score of 0.94 ± 0.04

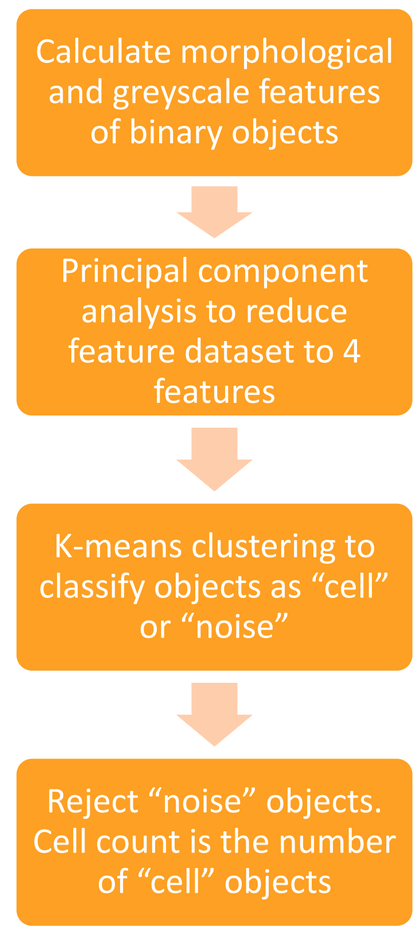


Figure 1 – binary object classification workflow

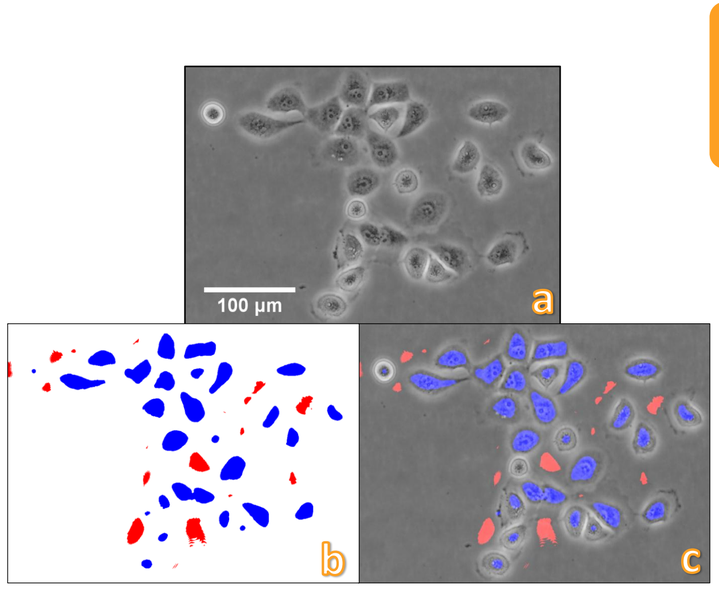


Figure 2 – a) low density image of keratinocytes. b) segmented image in which incorrect regions have been classified as "noise" (red) or "cell" (blue) using the method described. c) segmentation overlaid on original for comparison

Application and Results

Foetal calf serum supplementation

- Immortalised H400 keratinocytes [3] were seeded in 35mm culture dishes at an initial number of 3.6 x 10^4 cells for imaging
- Duplicate cultures were supplemented with 10%, 6% and 2% foetal calf serum (FCS) and imaged at time points between 48 - 120 hours post seeding
- Cell numbers at each time point were evaluated using the method described above and growth curves generated (figure 3)
- These growth curves indicated that H400 keratinocytes proliferated in a dose dependent manner with FCS concentration

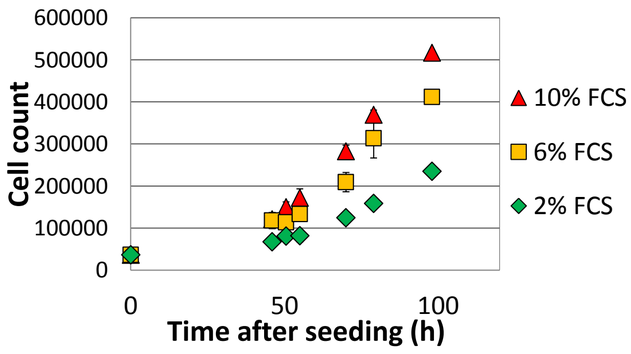


Figure 3 – growth curves of H400 keratinocytes supplemented with varying levels of FCS

Comparison with haemocytometer counting

- Additional cultures established under the same conditions were imaged and subsequently counted using an Improved Neubauer haemocytometer
- The image analysis method was systematically lower than the haemocytometer-measured count, although within one standard deviation (figure 4)
- The systematic operator error associated with haemocytometer counting could potentially have contributed to the discrepancy. Further work is required to determine the source of this difference

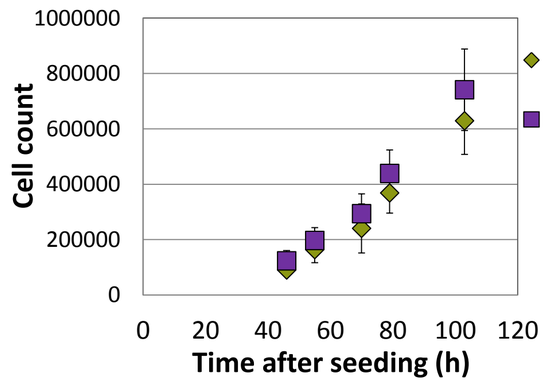


Figure 4 –growth curve determined using two enumeration methods.

Acknowledgements and references

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