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John Lawry is Honorary Secretary (Education) of the RMS and is a past Chairman of the Cytometry Section.

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He is joint organiser, with Mike Ormerod, of the annual Flow Cytometry Course (see page 45).

The DNA content of a cell can be readily measured using flow cytometry by allowing a fluorescent dye to stoichiometrically bind to the DNA molecule. As cells progress through the cell cycle they synthesise DNA, so if these cells are present they will take up more of the fluorescent dye and therefore fluoresce brighter than cells at an early stage of the cell cycle. Similarly, mixed populations of cells, such as are seen in tumour tissue, may well have a basal level of DNA different from that of normal tissue, owing to chromosome gain or rearrangement. They too will take up more of the fluorescent dye and be distinguishable as an additional, brighter population in the sample. The ratio of the peak position for the abnormal population to that of normal cells is termed the DNA Index, and is used in many cancers to aid diagnosis or predict outcome.

The flow cytometer is capable of rapidly determining the proportion of cells in the G1, S and G2/M phases of the cell cycle as well as relating the DNA content of the cell population to that of a suitable normal control. This form of analysis is widely used in many research laboratories, and also as a routine clinical assay for certain cancers, either as an aid to diagnosis (e.g. neuroblastoma, multiple myeloma, hydatidiform moles), or as a follow-up assay for minimal residual disease and response to treatment (e.g. lymphoma and leukaemia).

Sample selection

Flow cytometers require all samples to be uniform single cell suspensions. A suitable sample may be available in this form (blood sample, lavage, cerebral spinal fluid, peritoneal or bladder washing, suspension cell cultures), although clumps of cells may be present which will need to be dissociated. More frequently, the sample will be in the form of solid tissue. DNA measurements can be made from fresh, frozen or fixed tissue samples, the latter being technically the most challenging. Fine needle aspirates, brush and smear specimens (cervical, buccal, etc.) fall between the two sample types as they constitute either a narrow core of cells or a scrape of surface mucosa/epithelial cells, and usually contain clusters of cells which need to be vortexed in saline or media to produce a cell suspension.

Sample preparation

1. Mechanical dissociation

Place the tissue on a plastic Petri dish and dice with 'crossed scalpels' until the sample is reduced. Add 0.5 ml phosphate buffered saline (PBS) or tissue culture medium during the process. Place the sample on a gauge 60 stainless steel mesh and press the tissue through with a gentle rotary action using the end of a syringe plunger (a 1 ml syringe is best). Wash the dissociated cells out of the mesh using PBS or medium and pipette the released cells into a centrifuge tube using a plastic pipette. Spin at 400 g for 5 min. Tip off the supernatant and wash again in 5 ml PBS. Keep the reagents cold.



2. Automated mechanical dissociation

Cut the tissue into small pieces in a plastic Petri dish and insert into the Medicon cassette (see Figure 1). Allow the Medicon (from DAKO or BD) to dissociate the tissue for 10-30 s. Add 1 ml PBS or media to the cassette and dissociate for a further 10 s. Aspirate the cell suspension using a plastic pipette or needle and syringe and transfer all cells to a centrifuge tube. Spin at 400 *g* for 5 min. Tip off the supernatant and wash again in 5 ml PBS. Keep all samples and reagents cold.



Figure 1. The MediMachine for automated tissue dissociation.

3. Enzymatic dissociation

Dice samples of fresh or frozen tissue (primary or metastatic tissue) with a scalpel in a Petri dish, adding 1-2 ml PBS containing 0.01 mg/ml collagenase (Type II). Pipette the tissue suspension into a 25 ml universal, make up to 5 ml with more of the enzyme mixture and incubate for 15-33 min at 37°C with frequent mixing.

If the supernatant becomes cloudy with released cells, decant the liquid and, if needed, add more enzyme solution. Using a 300 gauge stainless steel mesh (grid approx. 50 μ m squares) filter the suspension into a 10 ml centrifuge tube. Spin at 400 *g* for 5 min. Tip off the supernatant and wash again in 5 ml cold PBS.

4. Extraction of nuclei from fixed embedded tissue

When tissue has been fixed in formaldehyde, single cell suspensions can only be prepared using enzymatic dissociation. This material is usually embedded in paraffin wax for routine histological examination, so thick sections (30-50 μ m) need to be cut. The resulting suspensions will contain both whole and sectioned nuclei, and a reasonable amount of cellular debris. In contrast, standard

5 μ m sections will only contain sectioned cells and nuclei and will be impossible to analyse by flow cytometry.

Nuclei are extracted from the section after dewaxing and rehydrating down through the alcohols. A simple way to do this is to mount the thick section on a glass slide. Sections can then be scraped off the glass and placed in pepsin solution (0.5%, Sigma) in an acidic buffer (pH 1.5) for 15-60 min at 37°C depending upon the degree of sclerosis and the cellularity of the tissue. Nuclei are then harvested and washed twice in cold PBS (Hedley *et al.*, 1983).

Staining procedures

1. Staining fresh or frozen cells

- Take $0.5-1 \times 10^6$ cells, centrifuge to a pellet and discard the supernatant. Mix the pellet in its own volume.
- Add 100 μ l 0.2% Triton-X100 (in PBS) and mix for 1 min at room temperature.
- Add 300 μ l 50 μ g/ml propidium iodide (PI) (Sigma) made up in PBS and 50 μ l 0.1 mg/ml RNase (Sigma) made up in PBS with 0.1% azide, and mix. Incubate for 30 min at room temperature (protect from light) and then store samples on ice.

Note: This method uses Triton as a detergent to permeabilise cell membranes so samples can only be held for a short period before they degrade. RNase is included to eliminate the non-specific staining of fold-back single-stranded RNA, in addition to the specific double-stranded nucleic acid staining seen with intercalating dyes such as propidium iodide.

2. Staining fixed cells

- Fix the cell suspension in 1-2 ml 80% ethanol (held at -20°C), vortex and store at or below 4°C. Cells fixed this way can be stored indefinitely at temperatures below -20°C. Alternatively, use nuclei extracted from formaldehyde-fixed, paraffin-embedded tissue.
- Wash an aliquot of cells/nuclei twice in PBS and stain as above with 50 μ g/ml PI and 0.1 mg/ml RNase. Better results are obtained if samples are allowed to stain overnight at 4°C.

3. Kit-based staining methods

Several commercial kits are now available which are easy to use and have a reasonable shelf life. There are generally two possible forms, both of which are designed for use with fresh or frozen cells, but which also work well with fixed samples:

- A single stain (no wash) format where the PI dye and RNase are lyophilised in buffer. Tubes are reconstituted with the solution provided and the cell sample added. This procedure usually gives scatter profiles

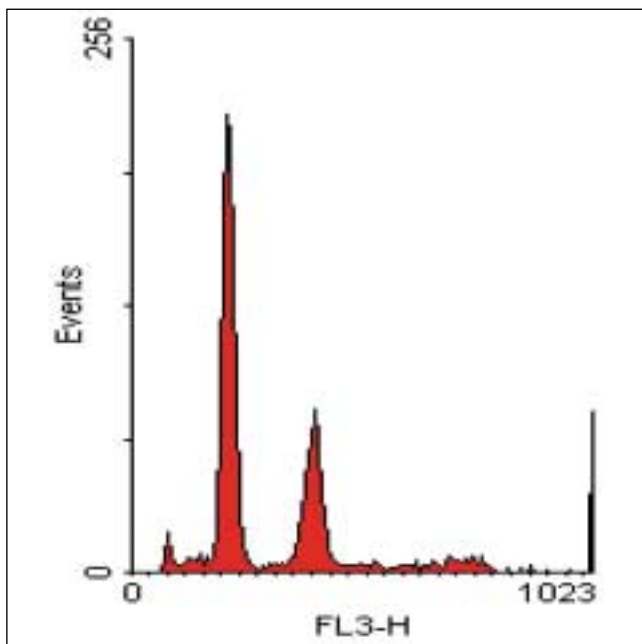


Figure 2. Adrenal mass containing 33% aneuploid cells, DNA Index 1.87. Diploid cv = 5.0, aneuploid cv = 4.0 Total S-phase = 10%. Stained using the Sigma DNA kit.

consistent with whole cells which have been detergent permeabilised before staining, e.g. DAKO Cons3 DNA staining kit.

- A three-stage assay where set amounts of three solutions are added sequentially, without any washing stages, to the sample. Typically the three solutions are trypsin, trypsin inhibitor with RNase, and PI. Scatter profiles from these samples indicate nuclear extraction and therefore reflect the method of Vindelov *et al.* (1983a), e.g. BD or Sigma DNA staining kit. An example of an adrenal mass stained with the Sigma kit is shown in Figure 2.

4. Staining viable cells

It is occasionally necessary to identify the cell cycle profile of intact viable cells, which are then allowed to continue to proliferate in culture. The above methods are all lethal to the cells because of the need to permeabilise the cell membrane to enable dye penetration. However, a limited group of dyes are able to penetrate the cell and nuclear membrane without prior permeabilisation. These include the bis-benzimidazoles such as Hoechst 33342. This drug binds to A-T rich regions of the minor groove of double-stranded nucleic acid, is excited by UV light and fluoresces blue. This of course can present a technical problem for cytometers not equipped with a UV light source. Staining is a one-step process taking 15 min.

Flow cytometric analysis

Samples

For all samples stained with a DNA dye, the most important consideration when analysing on a flow cytometer is the elimination of cell clumps and data

caused by multiple cells. The flow cytometer is 'blind', so acquires all data including that from, for example, two G1-phase cells stuck together, which will therefore have the same net DNA content as a single G2/M-phase cell. Clumps should be minimised before the sample is run on the cytometer by syringing through a 25 gauge (orange) needle two or three times, and/or filtering through 300 gauge stainless steel mesh which has a weave spacing of 300 threads per inch and a resulting mesh size of 50 μm (available from Rigby Wire, Sheffield and other sources).

Doublet discrimination

Doublet cells can be excluded on the cytometer by using fluorescence pulse shape analysis. Most cytometers enable two parameters, the pulse height and width, to be measured through the electronics of each fluorescence detector and from which a third parameter, the pulse area, is then calculated. As a cell passes through the laser light source, the fluorochrome emits its characteristic fluorescence. When the cell is in the centre of the laser the fluorescence reaches its maximum (pulse height); the time it takes the cell to pass through the laser completely gives the pulse its width. Samples are run at a constant rate so larger events (G2/M-phase single cells and G1 doublets) will have a larger peak area than singlet G1-phase cells, but the G1 doublet will also have greater peak width equivalent to the time it takes for both cells to traverse the laser.

Dot displays are therefore created for peak width versus peak area or peak height, depending upon the cytometer used. Gates are then set to surround the singlet G1, S, G2/M-phase populations, including any suspect aneuploid or endoreplicating cells, but excluding any cell doublets and cell debris. Frequency histograms can then be generated and the profile analysed. It is normal to employ specialist software to accurately assign areas within the histogram to designate each phase of the cell cycle, but a rough result can be made with simple marker settings. These stages of analysis are illustrated in Figure 3 for a sample of rhabdomyosarcoma.

Quality control

The accuracy in measuring the percentage of cells in each phase of the cell cycle will depend upon how narrow the main peaks are. The peak coefficient of variation (cv) is used to denote the quality of the sample and can be a good guide to the reliability of the percentage data. Samples would be designated poor if they had a cv above 8.0, which could be the result of insufficient staining times, too little dye or too many cells, fixation artefacts, as well as cytometer alignment problems, or simply running the sample through at too high a flow rate. Usually fresh or frozen tumour samples give a cv less than 5.0, whilst cell lines can yield a slightly higher cv.

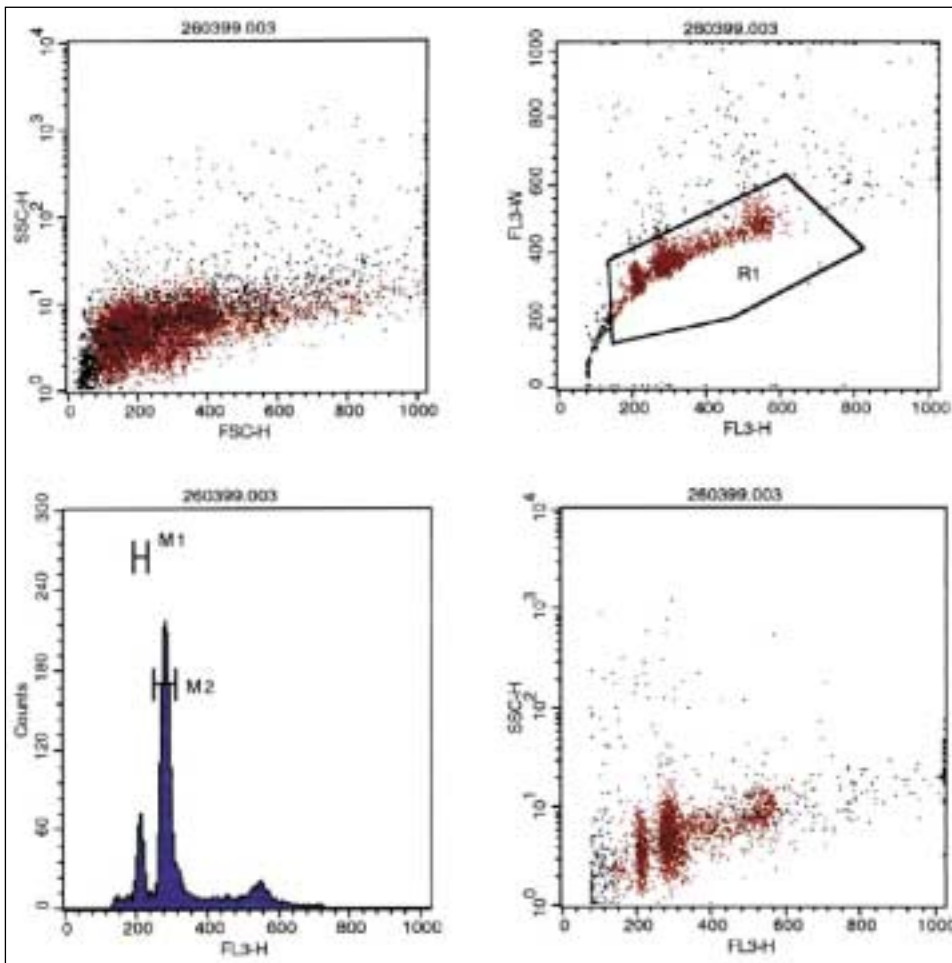


Figure 3. Rhabdomyosarcoma illustrating doublet discrimination gating (R1), containing 20% diploid and 80% aneuploid with a DNA Index 1.34. Diploid cv = 3.2, aneuploid cv = 3.4. Total S-phase=17%.

internal control is to add fixed chick and trout erythrocytes. They contain approximately 40% and 70% of human DNA respectively so form two discrete peaks to the left of any diploid G1 peak in the sample (Vindelov *et al.*, 1983b).

An external control would be a cell suspension which is stained and run in parallel to the clinical sample, as it enables the peak channel for normal diploid cells to be identified. A sample of separated lymphocytes is a good example as cells can be stored frozen in aliquots of 5×10^5 cells (in 10% serum and 10% dimethyl sulphoxide), or fixed in 80%

Software packages are of value in deconvoluting complex histograms where there are aneuploid as well as diploid populations which overlap each other. They also calculate the DNA index directly and provide information on the cv of the peaks as well as the ratio of the G1 to G2/M peak. These are all used to support the accuracy and validity of the data presented in the clinical report. A European Consensus report is a valuable reference document for those utilising clinical DNA analysis (Ormerod *et al.*, 1998).

ethanol and stored. The control sample can then be matched to the test sample (see Figure 4).

Controls

Experimental or research samples are usually in the form of cultured cells, so controls are usually cells grown under normal culture conditions. Cultures are clonal, so only a single cell cycle should be present. Drug-treated cells can show severely disturbed histograms so controls are valuable in determining the normal proportion of cells in each phase of the cell cycle. Some drugs are naturally fluorescent so may alter the relative fluorescence intensity of the DNA dye.

All clinical samples need normal controls. Normal cells (leucocytes, stromal cells, fibroblasts etc.) are usually in the sample mixture, and can be set as an internal control, but some tumours can be hypodiploid, so it is not always correct to assume that the left-hand peak is the G1 peak from normal cells. Dual staining with a leucocyte antibody (CD45) could resolve this issue as long as nuclear preparations are not used. Another form of

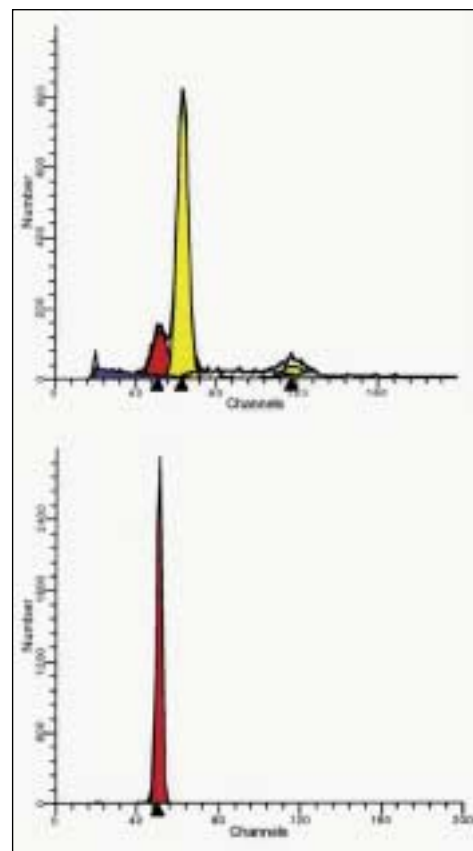


Figure 4. The value of the external diploid cell control (lower histogram) in determining the ploidy of populations within a tumour sample (upper histogram).

One type of sample which is hard to control accurately is nuclei extracted from formalin-fixed tissue. Formaldehyde cross-linking severely inhibits fluorochrome access to the nucleus, so staining levels are always around 50% less bright when compared with frozen or fresh nuclei. Variations may also exist within a tissue sample if it was inadequately fixed. Furthermore, different cell types within the sample can also show fixation and therefore staining variation. If control cells are fixed in formaldehyde they will not match the degree of fixation present in the tissue block so external controls are unreliable. This only leaves normal diploid cells within the sample for use as the marker population, and the hope that hypodiploid cells are not present. If a single population is seen it therefore has to be assumed to be diploid, but care must be taken in reporting results from these samples.

Conclusion

DNA cell cycle analysis is a simple yet effective tool for many areas of research. The method can be combined with antibodies to evaluate cell cycle related protein expression, and almost every cell type can be analysed, including bacteria and yeast.

The clinical use of DNA cell cycle and ploidy analysis is restricted to a small number of specific cancers in pathology laboratories within the UK, but is more widely reported elsewhere. Recent developments suggest a renewed importance of these measurements when monitoring a patient's response to treatment during

follow-up periods, as well as for monitoring minimal residual disease, so a technique that has been available for 20 years may be making a comeback as an aid to the treatment of cancer.

Acknowledgements

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