The use of 4, 6-diamidino-2-phenylindole, dihydrochloride (DAPI) in multiple flow cytometry applications

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Flow cytometry can be defined as a semi-automated procedure for the interrogation of single cells in a continuous fluid stream enabling the derivation of simultaneous measurements of multiple extra and intra cellular characteristics. The objective of flow cytometry is very simple; to measure, by quantitation of photon release, constituents of the membrane, cytoplasm and nucleus of a particular cell or group of cells.

This very important technique enables us to study the phenotype, genotype and cytoplasmic constituents of cells and bacteria. The cell analysis is very rapid, can be up to 20,000 cells per second, and is also multifactorial, whereby it has been reported that up to 17 probes, primarily fluorochrome conjugated antibodies (Perfetto *et al*., 2004), can be detected simultaneously on a single cell.

In addition, deoxyriboenucleic acid (DNA) specific dyes can be analysed either to define cell cycle characteristics or to be used in conjunction with surface markers. Historically propidium iodide has been widely used as a DNA specific dye, however, 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) is an excellent alternative dye especially with the current modifications to the laser configurations in bench top instrumentation.

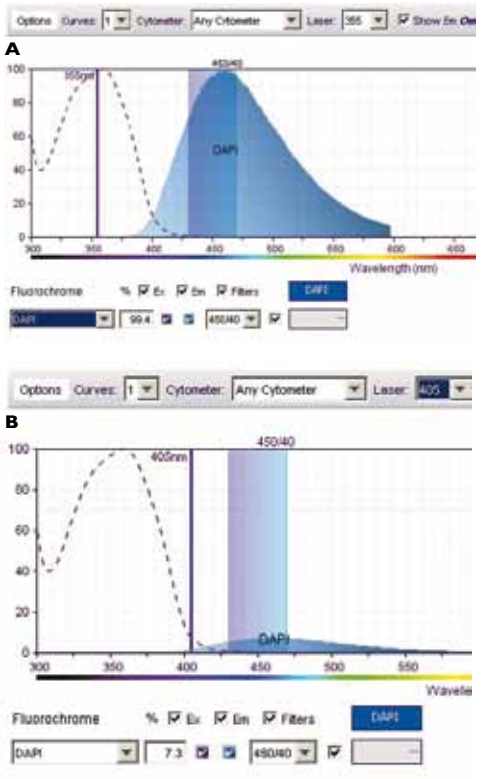


Figure 1: The laser line within the figure represents the excitation wavelength for both UV (A) and violet lasers (B). As can be seen in terms of the excitation by the violet laser, it is minimal (7.3%) as opposed to the excitation by the U.V. laser (99.4%).

Flow cytometers, both non-sorting and sorting platforms, are being developed to give an ever increasing capacity to look at new fluorophores and dyes, primarily because of the availability of cheaper, smaller solid state lasers and the advances of electronics and increasing market demands.

The most common and popular upgrade is that of the violet (405 nm) laser. The ultraviolet (UV) (355 nm) laser still remains quite expensive, although invaluable for using Hoechst 33342, a cell permeable dye that allows the visualisation of stem cell side populations. These cells can be identified based on their ability to efflux several vital dyes including Hoechst 33342. This ability to efflux dyes has been reported to be due to the expression of members of the ABC transporter family and will result in a typical and immediately recognisable staining reaction. The UV laser is also very useful for the detection of cell bound fluorophores, such as Alexa 350 and Quantum dots.

The violet laser gives many benefits in that fluorophores such as V450, V500, Pacific blue, BV421 and Krome Orange can be used for phenotyping, increasing the number of probes detectable simultaneously on the target cell population and therefore proportionately increasing the amount of data available with respect to the sample under analysis.

Importantly, and very conveniently, some dyes such as Hoechst 33342 and also DAPI can be excited by the violet laser, although on first viewing using a web based spectral viewer (www.bdbiosciences.com/research/multicolor/spectrum_viewer/index.jsp) this is not obvious. The excitation maxima quoted for DAPI is 358 nm and emission 461 nm. As can be seen in figure 1, the excitation by ultraviolet 355 nm laser and 405 nm lasers is very different, with respect to the excitation curve of DAPI, however, both lasers can be successfully used when analysing DNA by both Hoechst 33342 and DAPI.

This graphical representation of the excitation of both dyes is accurate but misleading, due to the fact that DAPI has a very high quantum fluorescence when bound to double stranded deoxyribonucleic acid (dsDNA). Therefore, even under conditions of sub-optimal excitation by the violet laser, the fluorescent product of DNA and DAPI will give an accurate DNA profile. DAPI binds preferentially to dsDNA associating with adenine thymine clusters within the minor groove of DNA. This binding will produce a 20 fold enhancement of DAPI fluorescence, emitting at 460 nm, in part due to the displacement of water molecules from DAPI and the minor groove of DNA.

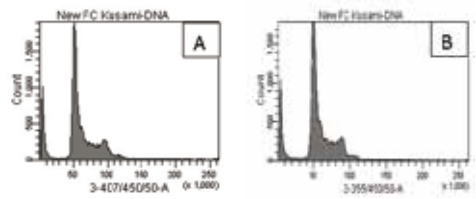


Figure 2: Histograms A &B are the cell cycle from a promyelocyctic leukaemia excited by: A-407 violet laser (50 mw) and B-355 UV laser (60 mw) (please note nomenclature throughout this article for axis labelling is flow cells laser). In this instance, the threshold was applied to the forward scatter parameter. These measurements were performed on the same sample simultaneously on a FACSAria cell sorter, demonstrating very similar results using both excitation sources. Emission of the DAPI was measured using a 450/50 nm band pass filter in both instances.

DAPI will also bind ribonucleic acid (RNA), however, the binding is to adenine and uracil selective intercalation, the emission wavelength is 500 nm and the quantum fluorescence is only 20% of that of the dsDNA reaction. This gives a distinct advantage over dyes such as propidium iodide, which is a base pair intercalator to both DNA and RNA, thereby necessitating the cells to be treated with an RNA destroying enzyme RNAse prior to DNA staining to ensure specific DNA staining. Figure 2 shows a DNA cell cycle from a sample of cells from promyelocyctic leukaemia: the analysis was performed simultaneously using the UV and violet lasers on DAPI treated cells.

Within our core facility we use DAPI for 3 major applications:

1. Staining dsDNA for cell cycle studies.
2. Use as a specific counterstain for Apoptosis and other nuclear dye applications.
3. Staining for dead cell identification prior to acquisition/sorting.

All examples of staining were performed using the Partec CyStain DNA 2 Step kit, although DAPI can be bought as a single reagent. We prefer the Partec kit as it gives us a readymade DAPI solution, as well as reagents to isolate cell nuclei for exceptional DNA analysis.

Staining dsDNA for cell cycle studies

The staining protocol for all DNA procedures using this DAPI kit is very straightforward and quick. A nuclear extraction buffer supplied with the kit is used to strip the cytoplasm from the cells (15 minute incubation) and then the DAPI solution is added to the cells and extraction buffer. A further incubation of the combined solutions is carried out (15 min. to 24 hours) and then the sample is analysed by flow cytometry. The concentrations of DAPI and extraction buffer reagents are proprietary information. Figure 3 shows actively proliferating cells stained by DAPI using the violet 405 nm laser, figure 4 exhibits the very similar results obtained on cell cycle using violet and UV lasers.

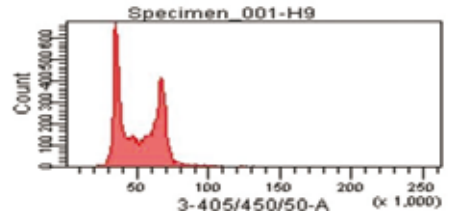


Figure 3: The above shows a typical example after 15 minutes with extraction buffer and 15 minutes DAPI solution; the cell cycle profile was achieved on a 40 mw 405 violet laser. A direct comparison can be made of results using a UV (355, 60 mw) or violet laser (407, 40 mw) below.

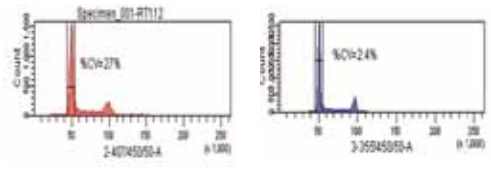


Figure 4: The coefficient of variation (CV defined as standard deviation of the mean divided by the mean) of the G0 peaks above demonstrate that the violet laser, in this instance, is giving a slightly wider CV (2.7%), this is not always the case. These histograms were produced after eliminating the doublets as defined by area and height pulse processing. These samples were acquired using the threshold specific for each dye.

To use as a specific counter stain for apoptosis and other nuclear applications

Apoptosis has two major functions:

1. Organised cell death to allow for the controlled renewal of that particular cell line.
2. The control of aberrant cell growth.

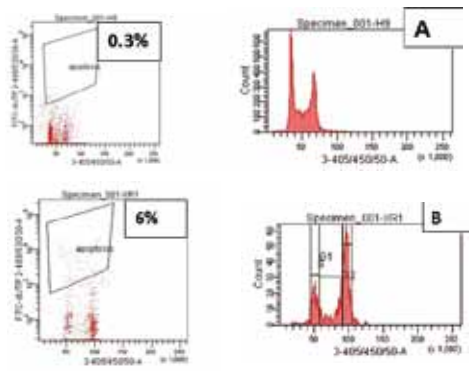


Figure 5: An example of low level of strand breaks with normal DNA profile, (B) example of increased G2 cell cycle with low level of strand breaks and (C) example of high level of strand breaks.

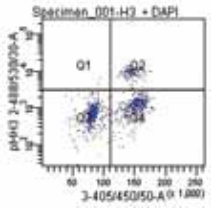


Figure 6: Left is an example of using DAPI as a DNA stain whilst simultaneously detecting histone H3 conjugated with fluoresceinisothiocyanate (F.I.T.C.). Histone H3 is low in interphase cells and occurs almost exclusively during mitosis as can be seen from this dot plot H3 is co localised with the G2 portion of the cycle analysis.

Apoptosis is characterised by loss of membrane asymmetry, condensation of the cytoplasm and nucleus as well as protein and internucleosomal cleavage of DNA, resulting in the elimination of the apoptotic cell by phagocytosis.

We have successfully used DAPI for the clarification of the cell cycle phase when determining Terminal Transferase dUTP Nick End Labeling (TUNEL) Assay for the detection of cleaved DNA strand breaks that are a hallmark of cells undergoing apoptosis, a programmed cell death.

This assay relies upon TdT catalysing the addition of bromolated deoxyuridine triphosphates (Br-dUTP) to the 3'-hydroxyl OH termini of double and single stranded DNA breaks. After the incorporation of Br-dUTP, a fluorescently conjugated antibody, BrdU will act as a reporter for the strand breaks. Figure 5 shows different levels of incorporated Br-dUTP whilst using DAPI as a DNA stain.

In addition to using DAPI along with dUTP, another application is the use of nuclear phenotypic markers as can be seen in figure 6 where a histone marker is clearly associated with G2 phase of cell cycle, as defined by the DAPI staining.

Dead cell identification prior to acquisition or sorting

DAPI does have the ability to pass through the membrane of live cells. However, the transit through dead cells is much more rapid thus enabling the differentiation of live and dead cell populations. Figure 7 shows a cell population prior to sorting without DAPI added and then cells from the same sample after the addition of DAPI.

There are a vast array of DNA and RNA probes: propidium iodide, ethidium bromide, hoechst, cyanine dyes such as Yo-Pro3, the Syto and Sytox dyes as well as the more commonly used 7-Aminoactinomycin D (7-AAD) and Draq 5. However, we tend to favour DAPI as a first choice because of its ease of use and relatively rapid and bright staining. In addition it is beneficial, if surface phenotyping is required, that there is an absence of excitation by the blue and red lasers, allowing simultaneous phenotyping to proceed using fluorophores reliant on blue and red laser excitation and emission channels.

With respect to sorting human leukemic bone marrow cells, an evaluation for colony forming assays using both Hoechst 33342 and DAPI was performed by Park *et al.* (1985). In an attempt

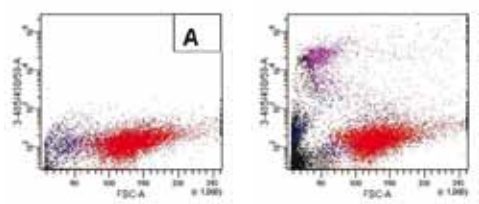
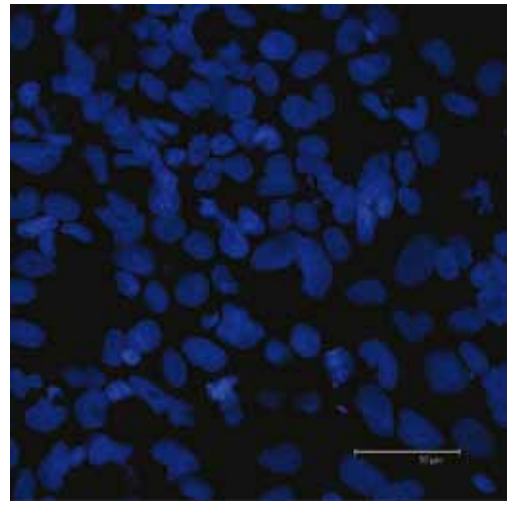


Figure 7: Sample A represents cells without the DAPI solution provided. To the right is the same sample after the addition of 50 ul of the DAPI solution.



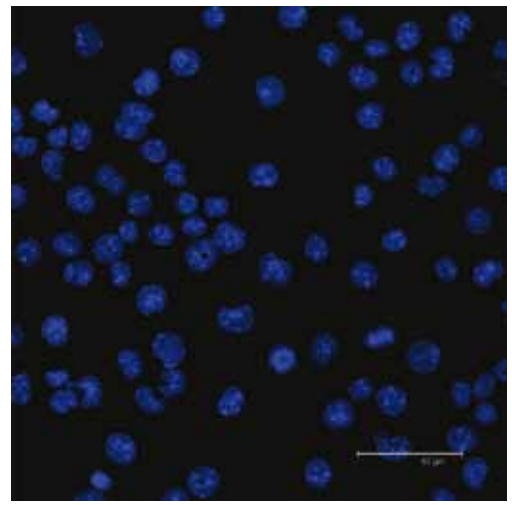


Figure 8 A and B: Micrographs showing DAPI stained nuclei imaged using the Coherent 360 nm broadband laser; the emission collected 400 - 510 nm.

to validate the toxicity of the two dyes, an initial evaluation verified optimal staining conditions for each dye was performed using Chinese hamster ovary (CHO) cells. Results showed slightly different staining methods were optimal for Hoechst 3342 and DAPI. In addition, DAPI gave better coefficients of variation for G0/G1 cells than did Hoechst 33342. This finding was also reproduced within mammalian bone marrow. It was shown that H33342 was more toxic than DAPI in terms of colony forming capability.

Conclusion

In summary, DAPI is a very convenient stain to use for multiple flow cytometric investigations, some of which I have outlined in this brief article.

The prerequisite is either a UV or violet laser as described earlier, both of which are becoming more common elements in everyday flow cytometers.

Acknowledgements

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References

Perfetto SP, Chattopadhyay PK and Roederer M. 17-color Flow Cytometry: Unraveling the Immune System. *Nature Reviews Immunology*, 2004 August; 4(8):648-55

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Ian Dimmick began his career in a clinical setting within the health service, focusing primarily on practical laboratory haematology and immunology, before progressing to lecture on both subjects within Newcastle and Northumbria Universities.

The role of flow cytometry in routine leukaemia, lymphoma diagnosis and HIV monitoring has became very important. The diversity of this very powerful tool for both clinical and research applications led Ian to develop an interest in flow cytometry and this afforded the opportunity to join Beckman Coulter, Dako and Becton Dickinson as a flow cytometry application specialist, where he tracked and developed the rapidly moving changes within this science.

Later, Ian returned to Newcastle University as manager of a busy flow cytometry core facility, where he has since faced many unique challenges; the assay of stem cells, multiplex assays and polychromatic flow cytometry being just a few.