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Minireview

Molecules and Cells

Use of DNA-Specific Anthraquinone Dyes to Directly Reveal Cytoplasmic and Nuclear Boundaries in Live and Fixed Cells

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Image-based, high-content screening assays demand solutions for image segmentation and cellular compartment encoding to track critical events - for example those reported by GFP fusions within mitosis, signalling pathways and protein translocations. To meet this need, a series of nuclear/cytoplasmic discriminating probes have been developed: DRAQ5™ and CyTRAK Orange™. These are spectrally compatible with GFP reporters offering new solutions in imaging and cytometry. At their most fundamental they provide a convenient fluorescent emission signature which is spectrally separated from the commonly used reporter proteins (e.g. eGFP, YFP, mRFP) and fluorescent tags such as Alexafluor 488, fluorescein and Cy2. Additionally, they do not excite in the UV and thus avoid the complications of compound UV-autofluorescence in drug discovery whilst limiting the impact of background sample autofluorescence. They provide a convenient means of stoichiometrically labelling cell nuclei in live cells without the aid of DMSO and can equally be used for fixed cells. Further developments have permitted the simultaneous and differential labelling of both nuclear and cytoplasmic compartments in live and fixed cells to clearly render the precise location of cell boundaries which may be beneficial for quantitative expression measurements, cell-cell interactions and most recently compound *in vitro* toxicology testing.

The resolution and sensitivity of fluorescence-based microscopy now permits the development of biological and functional assays whereby the change is observed as the altered intensity, re-distribution or translocation of a fluorescently tagged protein species (e.g. with eGFP). Examples of these include Rac-1, β-arrestin, MAP Kinase, NF-κB and transcription factors such as FHKR.

Developments in automation and image analysis software have enabled such assays to be performed at relatively high throughputs, such that they can be contemplated for primary screening of compound and siRNA libraries often containing more than 10^6 members.

However, a number of challenges arise from the combination of these features to allow robust assay development. Given the normal (and often wide) distribution of cellular response to a stimulus it is helpful to identify a representative number of individual cellular objects of interest in a field of view. Analogous to this is the use of a fluorescent DNA dye to gate nucleated cells from non-nucleated cells and debris in flow cytometry of blood and bone marrow where the dye fluorescence is used as a "trigger" for events to be analysed. This permits better statistical evaluation, observation of well-to-well variations, plate "edge" effects and generalized cytotoxicity. Certain proteins have promiscuous expression in more than one compartment of the cell (e.g. between the cytoplasm and nucleus) which may make the establishment of baseline or threshold expression more difficult. In this case the use of a nuclear DNA staining fluorescent dye probe (commonly referred to as a "counterstain") can aid in the segmentation of nucleus from cytoplasm, commonly in use in immunofluorescence microscopy and FISH. This would allow the measurement of the cytoplasmic (or, for example, mitochondrial) signal to the exclusion of the nuclear one and *vice versa*. Such a fluorescence signal needs to be spectrally separated from that of the protein "reporter" either by excitation or emission, and the requirements for this will depend on the configuration of the imaging platform employed. For instance, although the semi-permeant DNA dye DAPI has an emission signature that overlaps with eGFP (analogous to FITC for immunolabelling and Cy2 for FISH), its excitation is achieved from a UV source whilst that of eGFP is from the blue part of the spectrum often using an ion-Argon source at 488 nm. Therefore, only sequential excitation will permit generation of the required (merged) image i.e. requiring two scans of the field of cells and thereby doubling the acquisition time. Alternatively, eGFP and the non-permeant nucleic acid propidium iodide (PI) can be co-excited with light at 488 nm whilst their emission signals can be separately detected by selection of appropriate filters. In practical terms, however, PI is not suited as a counterstain as the degree of RNA staining is such that the nucleus is not distinguishable in interphase cells, even following permeabilization and RNase treatment as shown in Martin et al. (2005). An ideal counterstain would also limit the possibility of FRET-like excitation of the reporter protein's fluorescent tag and it would not quench, interfere with or compete with the tag's signal. Importantly, the spectral properties of the assay and the

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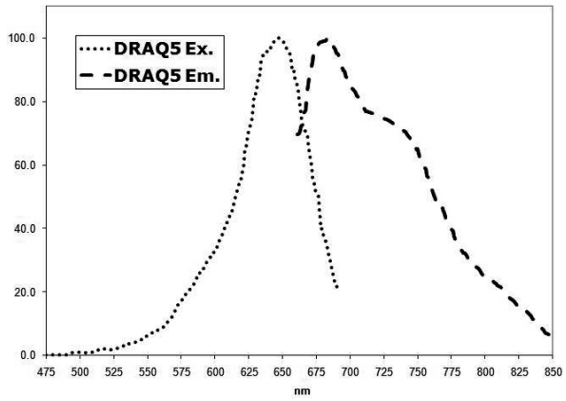

Fig. 1. Excitation (abs.) and emission spectra for DRAQ5

counterstain should permit transferability of a method from a development platform to a high throughput platform. As described by Smith et al. (2000) the fluorescent probe DRAQ5™ meets these primary criteria: it has high specificity for dsDNA, strongly binding the minor groove at A-T, A-T sites; its peak absorbance (excitation) is at 646 nm and its emission signal starts at 665 nm, peaking at 697 nm (DNA-bound) yet detectable into the far-red region (Fig. 1) separating it both by excitation and/or emission from the majority of visible range fluors and most importantly the protein reporter tags CFP, GFP, YFP and RFP as well as the antibody labels FITC, PE, PE-Texas Red and their analogues allowing rapid, parallel image acquisition for at least two parameters (e.g. nucleus and protein reporters); there is no evidence that DRAQ5™ (photo-)chemically interferes with the signal from another fluor by quenching or FRET-like mechanisms; and DRAQ5™ has been commonly used in cell-based assays and multi-colour flow cytometry as exemplified by Bjornsson et al. (2008) and is widely applicable to fluorescence microscopy, by Visconti et al. (2006), and HCS platforms, by Loechel et al. (2006) since most are equipped with a HeNe laser (635 nm) and a far-red detection channel (often associated with the Cy5 fluorescent label). An additional feature of a red-excited live cell permeant counterstain is that the excitation wavelength light penetrates deeper and with less scatter into tissue sections than for the previously described UV-excitation (with dyes such as Hoechst 33342 and DAPI) whilst the emitted light moreover, further red-shifted, similarly benefits from this effect.

Having chosen a suitable nuclear counterstain, image analysis software can then be applied to segment the nucleus based on this staining. Algorithms have been developed which “dilate” radially from the nucleus to provide an assumed cytoplasmic zone around the nucleus. Tools to achieve this are provided by the majority of high-throughput automated imaging platforms and by independent image analysis software suppliers (e.g. Definiens AG’s Cellenger product).

However such an approach requires a conservative zone to be selected within the cytoplasm that can be measured for any given change. At the very least this can affect the statistical value of the measurement and at worst may render the datapoint invalid if the area chosen for measurement overlaps a large sub-compartment such as a mitochondrion or where the cytoplasmic compartment is significantly heterogeneous in shape, size or volume across the cell population. This is clearly less of an issue where there is always some expression of a cytoplasmic signal since this will delineate the compartment but

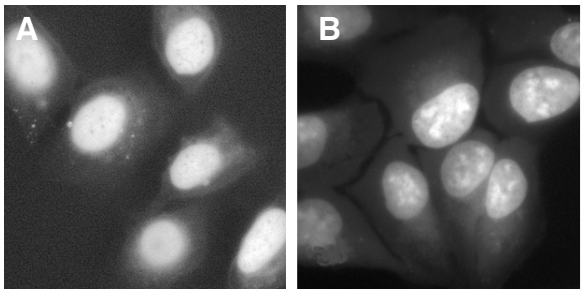

Fig. 2. Live U2-OS cell staining. (A) DRAQ5, (B) CyTRAK Orange

this will require adjustment of detection threshold or parameters to account for reference or basal level which might limit the dynamic range. This is unwelcome where the stimulus applied to the cells causes significant changes in cytoplasmic distribution or “texture”, for example via different endocytotic pathways as described by Pelkmans et al. (2005).

One solution might be afforded by use of a combination of probes to demark the two compartments as used by Cogger et al. (2008). The difficulty is finding two probes that have similar spectral properties (to avoid occupying too much of the available spectrum), require little titration between different cell types and which can be used in either live or fixed cells. Further, the addition of another molecule into the analytical complex raises the risk of chemical interference, quenching and limits cross-assay and cross-platform compatibility.

It would be advantageous, then, to have a nuclear counterstain which operates with a single spectral profile, separated from the common visible fluorescent reporters and antibody-/FISH probe-tags yet which has a useful and differential staining intensity for and between the nucleus and the cytoplasm. This would perhaps be achieved where the DNA binding dye also exhibits a low level of promiscuity in binding to dsRNA. Excitation by a red wavelength (e.g. 633 or 647 nm) is preferable as this limits the background signal contribution due to biofluorescence and avoids local heating that might be encountered when detecting weak signals with a UV laser, typically with a much higher energy output. Likewise, a nuclear counterstain with high chemical and photo-stability (low photobleaching) would be advantageous. Again, DRAQ5™ meets these needs. There is a demonstrable and appropriate level of cytoplasmic staining to allow segmentation of that compartment in addition to the much brighter staining nucleus (Fig. 2A). It is a red-excited dye, well separated from the visible range fluors. DRAQ5™ shows excellent chemical and photo stability in aqueous solution at ambient temperatures and, as shown by Martin et al. (2005), very low photo-bleaching compared to several other fluorescent molecules.

Cell-based high content screening experiments can be broadly separated into two types; with either fixed or dynamic endpoints. Where the change is temporally short-lived or unstable it may be beneficial to fix cells to allow imaging to take place independent of the end-point. However, where in the work of Foley et al. (2005) the reporter molecule leaches from one compartment to another or even out of the cell in the case of sub-G1 apoptotic DNA fragments (Fig. 3) upon fixation or permeabilization there is then a pressing requirement for a live-cell membrane-permeant agent. Such a requirement would also hold for dynamic end-point assays such as those used for a Rac-1 translocation assay system developed by Amersham

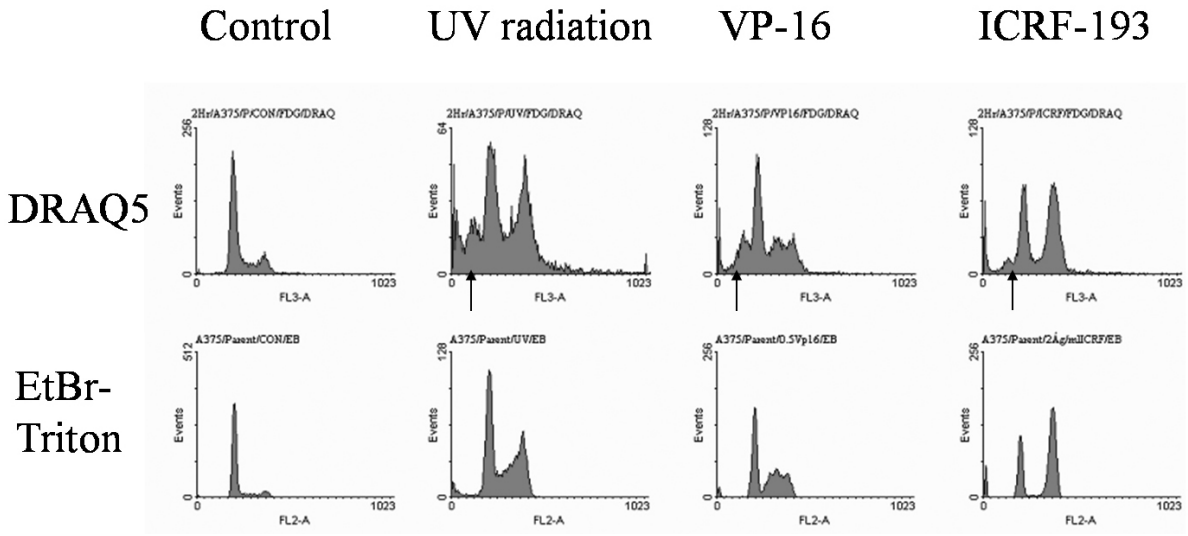

Fig. 3. The impact of cell permeabilization on the wash-out of sub-G1 fragments in induced apopotosis

Biosciences (2003) where perhaps a wide natural variation in the control setting (i.e. prior to stimulus) might need to be accounted for. Equally, it might be that initially a cell-based assay moves from a dynamic end-point to a fixed end-point when implemented in the routine for high throughput application. Thus, for ease of assay development the ideal agent should perform equally well on both fixed and live cell preparations with the minimum of titration and, helpfully, function in normal physiological buffers, culture media and, if possible, in pre-fixation, post-fixation and "in-fixative" modes. It is essential that a dye used on live cells labels in a temporally-stable manner since the need for a timed addition of that component to an assay procedure would be an unwelcomed complication, particularly when reading a microtitre plate that might have 1536 wells. In essence, this would require a dye probe with high avidity/affinity for the target DNA rendering it "invisible" to ABCG2 pumps most often associated with drug clearance from cells (especially the case for multi-drug resistant phenotypes in primary cancer cells or tumour cell lines) unlike the UV excitable, live cell DNA dye Hoechst 33342 which is progressively effluxed due to the highly dynamic nature of its binding to DNA, used as a reference for efflux of other drugs in a study by Garcia-Escarp et al. (2004), and notwithstanding its unhelpful emission overlap with GFP, FITC and equivalent fluorophores. At a practical level, an agent which is supplied ready-to-use (i.e. water-soluble) and which does not require a washing step would be beneficial in automation and the streamlining of experimental design. Lastly, the staining should be at a satisfactory level in a matter of minutes.

DRAQ5™ is highly lipophilic and readily crosses cell and nuclear membranes in intact live cells and tissues to bind to DNA, according to Smith et al. (2000). It has also been widely used on fixed tissues and cells, prepared with a variety of fixatives as well as prior to fixation and in fixative, as used by Haasen et al. (2006). DRAQ5 exhibits excellent temporal binding stability in live cells over many hours as clearly demonstrated by Martin et al. (2005) and Rosado et al. (2008) and is not noticeably effluxed by cells which have either over-expression of ABCG2 as in the work of Garcia-Escarp et al. (2004) or demonstrable (multi-) drug resistance. Cell staining (live or fixed) with DRAQ5™ is effected in a few minutes rapidly reaching a stable equilibrium that so favours DNA that washing is not required since the low concentration of dye in the surrounding medium is typically below the threshold of detection. Despite being lipophilic, DRAQ5™ is readily soluble in aqueous solution, is commercially supplied thus, and, importantly, is compatible with buffers such as PBS and cell culture media.

The basic property of an ideal agent described thus far is dependent upon its highly preferential DNA binding. If this labelling was stoichiometric to DNA then it would be possible to take further advantage of the segmentation of the relatively bright signal in the nucleus. Once segmented that signal could report DNA content (cell cycle position) in that individual cell. Doing this for every cell would then permit cell cycle analysis for the whole field in view. This could be important in study of anti-cancer agents, inflammatory response, and so on or where the population was heterogeneous in its response to the given stimulus. Such an approach was recently demonstrated by May et al. (2008) using a PerkinElmer/Evotec Opera™ high content imaging platform. Ideally, this property should be exhibited in both fixed and live cells and be most readily demonstrated by flow cytometry. Where fixation is required or preferred then the choice of fixation and permeabilization should be carefully evaluated since solvent-based methods favour DNA analysis whilst cross-linking methods favour proteins. In this context DRAQ5™ shows excellent stoichiometry with DNA content in both live and fixed cells (Fig. 4). This is aided by the fact that DRAQ5™ is a non-enhancing dye, does not exhibit enhanced fluorescence upon binding to DNA and therefore DNA-bound dye intensity reflects DNA content with good linearity. DRAQ5™ has been widely exploited to report DNA content (cell cycle position, sub-G1 peaks indicating apoptosis, and shifted G1 peaks pointing to aneuploidy) in blood, bone marrow and lymph node samples by flow cytometry by various laboratories including Plander et al. (2003), and Yuan et al. (2004), Primo et al. (2006), Swerts et al. (2007) demonstrating its applicability.

This stoichiometric labelling by a suitable DNA probe dye can also be applied to whole well or so-called "wellular" analysis. The total DNA signal from a field of diploid cells is directly proportional to the number of cells and can be used to normalise

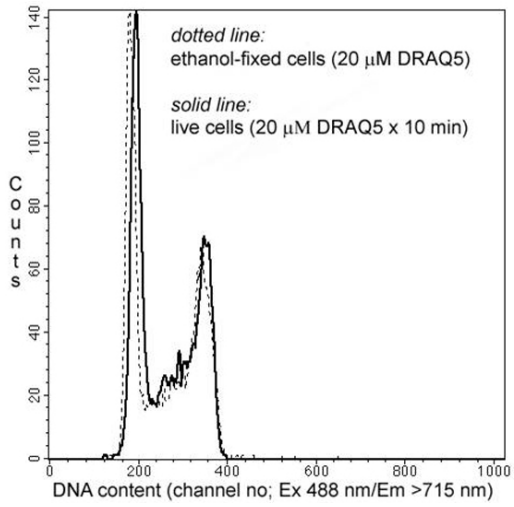

Fig. 4. Flow cytometric DNA content measurement in live or fixed cells shows that the staining remains stoichiometric in both cases.

the labelled antibody signal, thereby taking into account the well-to-well variations in the contributing cell numbers. DRAQ5™ has been applied in this manner for so-called "in-cell western" experiments performed by Richardson et al. (2008) and Hannoush (2008) in high throughput mode on the Odyssey™ Infrared Imaging System, LI-COR Biosciences showing the excellent linearity of DRAQ5™ dye signal to cell number. Interestingly, another recent report by Laakkonen et al. (2007) describes the impact of baculovirus transduction on distribution of chromatin in the human hepatocyte cell line HepG2. DRAQ5™ was used to show the dispersion of chromatin resulting from transduction, confirmed by monitoring histones. Again, these data support DRAQ5™ as a highly effective and faithful reporter of DNA/chromatin in living eukaryotic cells.

The place of drug safety has grown in importance in recent years. Clearly drug manufacturers want to avoid unsafe compounds reaching the marketplace but they also want to incorporate toxicological "filters" earlier in the drug discovery process, even in primary/secondary screening, to limit the unwanted and exponential costs when the toxic effects of a compound are only identified at a later stage in the development pipeline. The recent adoption of high content screening technologies further enables this. At its simplest, a reduction in the number of nuclei in a field would infer that a compound is having a catastrophic effect on cell viability. A fluorescent DNA probe dye would report this. In an elegant method for identification of AKT-signalling pathway inhibitors developed by Rosado et al. (2008) DRAQ5™ was used as the reporter of both the relative cell numbers between a mixture of isogenic cells genetically-engineered to be sensitive and insensitive to specific inhibition and also the unwanted generalised cytotoxicity resulting in the decrease of both sub-populations. A similar strategy has been used by Simonen et al. (2008) to screen out toxicants from those compounds which inhibit prenylation. However, in addition to absolute cell numbers, cells were also monitored for any significant increase in nuclear DRAQ5™ fluorescence intensity resulting from nuclear condensation. When one of these two features was seen to the exclusion of the other then a different mode of toxicity was inferred. Using a DNA probe with ideal qualities of the kind described earlier would potentially offer further morphometric information on the impact of a compound on a cell since one could now measure the change in shape or area of the nuclear and the cytoplasmic envelopes. Such an approach has been used to evaluate the unwanted cytotoxic effects of anti-viral compounds against host/target cells in an HIV infection inhibitor assay by Gustin et al. (2009). In essence a combination of the degree of change of nuclear roundness, and cell area and cell roundness were found to be robust indicators of unwanted cytotoxicity. The measurements of each of these parameters relied solely on the properties of DRAQ5™. Similarly, it is possible to detect micronuclei, nuclear fragmentation or condensation events. As mentioned earlier, cells can be analysed for a compound's impact on the progression of the cell cycle while higher resolution imaging permits further studies of the structural changes to the chromatin. Such an approach was developed to study the redox status of cells (detecting glutathione [GSH] by monochlorobimane, reactive oxygen species [ROS] by H_2DCFDA) in different phases of the cell cycle (i.e. by DRAQ5™ labelling) using flow cytometry by Conour et al. (2004). Using this panel of three functional probes with the addition of a fourth for mitochondrial membrane permeabilization (TMRM) Xu et al. (2008) have shown the ability to report cytotoxicity of lead compounds, in a four-colour cell-based imaging assay, has been further extended. Using primary human hepatocytes they were able to strongly correlate the results to a large panel of compounds with historically well described and classified hepatotoxicity ranging in severity and the point of their removal from the development process or the market. One fascinating revelation of the work was the observation that the DNA probe DRAQ5™ exhibits a useful labelling of peri-nuclear lipid vesicles, indicative of drug-induced phospholipidosis in hepatocytes. Although this might not be surprising since a live cell-permeant probe might be expected to be lipophilic in nature, such a phenomenon has not been observed with other cell permeant DNA probe dyes and this remains an important feature of hepatotoxicity.

Such measurements would add to the enumeration of cells and cell cycle analysis for the population whilst, with the optimal set of features described earlier would also permit multiparameter analysis using other fluorescent functional probes, reporter proteins and labelled antibodies.

Recent developments

There are further challenges in cell-based assays requiring the development of functional probes which more brightly show the full extent of the cytoplasmic envelope whilst retaining the ability to differentially segment the cytoplasm and nucleus. This would be very useful where one of the translocation termini was the cytoplasmic membrane. To meet this requirement, CyTRAK Orange™ was developed by Errington et al. (2006) by a chemical modification of DRAQ5™. It has significantly more promiscuous binding to RNA and thus labels the cytoplasm more brightly than DRAQ5™ permitting visualisation adjacent to the cytoplasmic membrane (Fig. 2B). Like DRAQ5™, CyTRAK Orange™ is live cell permeant but due to its high RNA binding loses the ability to report DNA content (except in RNase-treated cells). Its spectral profile (Exλ_{max} 510/Emλ_{max} 610) allows co-excitation with GFP but importantly retains detection in separate channels. This makes it an ideal counterstain for early live-cell epifluorescent microscopes as these often did not allow far-red detection or where the far-red detection is unsatisfactory. Here the co-excitation avoids problems of pixel/pixel registration and laser alignment. CyTRAK Orange™ should be an excellent agent for use with the Cellomics ArrayScan™.

Summary

The live cell DNA binding dye DRAQ5™ identifies the objects of interest in a fluorescence microscopy field of view i.e. nucleated cells. This then allows enumeration of the number of surviving cells compared to the control - giving one measure of cytotoxicity. Segmenting the brightly stained DRAQ5™ positive nuclei, the DNA content (cell cycle) profile of the population in a field of view can be plotted: for anti-proliferative effects; for apoptosis and for disturbance of cell cycle progression. Similarly, small yet equally bright DRAQ5™ labelled micronuclei can be detected proximal to the nucleus. Two-compartment (cyto:nucl) segmentation using DRAQ5™'s differential staining pattern provides the cell texture for the effective study of many translocation events and enhances the performance of software-based segmentation tools. Moreover, this segmentation allows the development of morphometric analyses where nuclear and/or cytoplasmic shape and area changes occur upon cheical "insult" by a candidate drug compound. The recent finding that DRAQ5™ demarks phospholipidosis in hepatocytes adds a new and valuable parameter in the drug discovery process.

It is perhaps remarkable that DRAQ5™ can provide all of this valuable information while meeting the other key requirements for fluorescent microscopy cell-based assays: spectral separation from the commonly used visible range fluors; non-quenching/non-FRETing; temporally stable; ABCG2 or MDR phenotype insensitive; chemically stable; photo-stable with low photobleaching; water soluble for buffer compatibility; equally staining live cells or fixed cells; and highly transferable across different imaging platforms including hybrid imaging cytometers (e.g. ImageStream, Amnis Corp.) as shown by George et al. (2008) and plate based cytometers (e.g. Acumen eX3, TTP Labtech) by Payne et al. (2007). Meanwhile, as described, the recently developed CyTRAK Orange™ offers additional resolution at the cytoplasmic boundary as well as labelling the nuclear and cytoplasmic compartments and is conveniently co-excited with GFP.

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