

## The Analysis of Cell Cycle, Proliferation, and Asymmetric Cell Division by Imaging Flow Cytometry

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### Abstract

Measuring cellular DNA content by conventional flow cytometry (CFC) and fluorescent DNA-binding dyes is a highly robust method for analysing cell cycle distributions within heterogeneous populations. However, any conclusions drawn from single-parameter DNA analysis alone can often be confounded by the asynchronous nature of cell proliferation. We have shown that by combining fluorescent DNA stains with proliferation tracking dyes and antigenic staining for mitotic cells one can elucidate the division history and cell cycle position of any cell within an asynchronously dividing population. Furthermore if one applies this panel to an imaging flow cytometry (IFC) system then the spatial information allows resolution of the four main mitotic phases and the ability to study molecular distributions within these populations. We have employed such an approach to study the prevalence of asymmetric cell division (ACD) within activated immune cells by measuring the distribution of key fate determining molecules across the plane of cytokinesis in a high-throughput, objective, and internally controlled manner. Moreover the ability to perform high-resolution, temporal dissection of the cell division process lends itself perfectly to investigating the influence chemotherapeutic agents exert on the proliferative capacity of transformed cell lines. Here we describe the method in detail and its application to both ACD and general cell cycle analysis.

**Key words** Cell cycle, Asymmetric cell division, Cell proliferation, CellTrace Violet, Dye dilution, Mitosis

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### 1 Introduction

In all higher organisms, genetic fidelity and tissue homeostasis is maintained by exerting tight molecular controls over cell cycle commitment, progression, and exit. If a single cell accumulates multiple loss or gain of function mutations that affect these controls then this can lead to oncogenesis [1, 2]. In the majority of cases, successful completion of the cell cycle generates two quantitatively and qualitatively matched daughter progeny because any pre-existing macromolecules (DNA, RNA, protein) or pre-formed structures (endosomes) are apportioned equally across the plane of

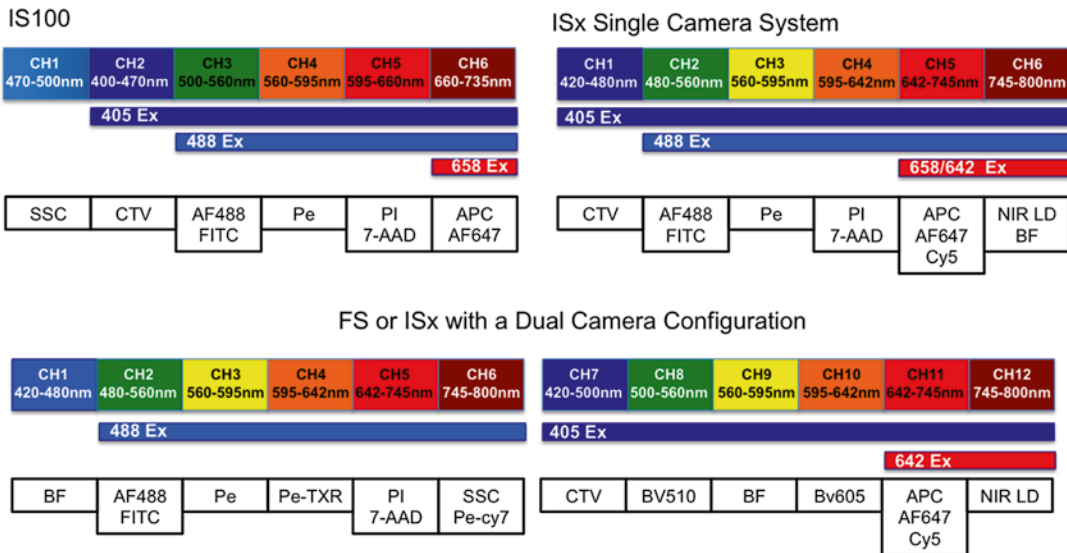
cytokinesis. However in certain biological systems these key elements are not distributed in a symmetrical fashion leading to what is described as an asymmetric cell division (ACD). ACD has been shown to play a key role in cellular differentiation in developmental and stem cell biology [3–5]. It has also been suggested to play a role in the adaptive immune response to direct the development of effector and memory populations [6–10] although this remains highly controversial and unsupported by various studies that have elegantly tracked the immunological fate of single cells [11–15]. Any technique designed to determine the prevalence and role of ACD must firstly be capable of providing multi-parameter fluorescence-based imagery that can be measured on a quantifiable (relative) scale. Secondly it should be capable of high-throughput sample acquisition in order to identify statistically relevant numbers of rare, short-lived mitotic phase cells without the need for any enrichment using chemical inhibitors. Thirdly there should be a temporal component to the method that is able to cope with the asynchronous nature of cell proliferation, even within seemingly homogenous lymphocyte populations. Lastly there should be an objective and controlled analysis framework whereby asymmetry is identified/scored based on some kind of known internal control limit for symmetrical inheritance. To this end we recently described a novel method for reporting the cell cycle position and division history of asynchronously dividing cells using CellTrace Violet (CTV) fluorescence dye dilution, propidium iodide (PI) for DNA content, and co-staining for mitotic phase antigens such as MPM2 [16]. When used in conjunction with an imaging flow cytometry (IFC)-based instrument platform, it is able to provide the spatial information to subdivide mitosis into prophase, metaphase, anaphase, and telophase to then measure the distribution of various key fate determinants across the cytokinetic plane. This has been successfully employed to study ACD in both T and B cells responding to antigenic stimulation [12, 17]. As a secondary but equally powerful function, our method can also be used to study the role of chemotherapeutic agents on transformed cell lines to determine where and how they influence cell cycle progression. The method is relatively simple to set up using virtually any cell type and in this chapter we describe the protocol in detail so that it can be reproduced with additional notes covering the major issues and pitfalls.

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## 2 Materials

2.1. *Essential cytometry hardware.* The minimum IFC instrument (Amnis, Seattle) requirements are one from the following (see Fig. 1):

- ImageStream 100 (IS100): 10-bit CCD camera, dynamic range 0–1024, 6 camera channels, and up to 5



**Fig. 1** The spectral layout of four different IFC systems we have successfully used to implement versions of our method. In all cases, the excitation lasers and approximate emission channel widths are shown with major fluorochrome examples to aid panel design. Although the IS100 offers limited flexibility, we have successfully tracked division with CTV, identified mitotic cells with PI/PH3 and measured one fate-determining signal within these cells [17]. The newer generation systems offer greater experimental flexibility and data content

of these can be fluorescent stains plus side scatter, fixed at 40× magnification.

- FlowSight (FS), 12-bit CCD camera, dynamic range 0–4096, 12 camera channels up to 10 of which can be used for fluorescent stains, fixed at 20× magnification.
  - ImageStreamX MKI or MKII (ISXMKI/II): 12-bit CCD camera, dynamic range 0–4096, 6 or 12 camera channels of which 5/10 of which can be used for fluorescent stains depending on configuration, 20, 40, or 60× magnification with multi-mag upgrade otherwise fixed at 40×.
- All systems should possess 488 nm, 405 nm, and 654/642 nm excitation lasers, with a 561 nm laser optional (not available for the IS100).
  - All systems should be fully ASISST calibrated with additionally quality control performed using a multi-level fluorescent microsphere set. Particular attention should be paid to the resolution of peaks 2 and 3 (dim 1 and dim 2) at laser powers used to excite experimental samples.

2.2. *A conventional flow cytometer (CFC) system* with an analogous excitation laser and detector configuration to the IFC systems listed above (*see Note 1*).

### 2.3. Media and buffers:

2.3.1. Primary T cell/Jurkat culture media: RPMI supplemented with 10 % fetal bovine serum (FBS), 100 units of penicillin/streptomycin, 2 mM glutamine, and 50  $\mu$ M 2-mercaptoethanol (2-Me).

2.3.2. B cell culture media: RPMI supplemented with 10 % FBS, 50  $\mu$ M 2-Me, 25 mM HEPES, 2 mM GlutaMAX, and 10 U/ml penicillin/streptomycin.

2.3.3. Wash/stain buffer: PBS supplemented with 2 % FBS.

2.3.4. Permeabilization buffer: Wash/stain buffer supplemented with 0.1 % Triton-X 100.

### 2.4. *CellTrace Violet*<sup>TM</sup> (Cat No A10198; Life Technologies):

There are a number of commercially available fluorescent dyes that are able to record the division history of single cells through dye dilution [18]. In general terms there are two categories of reagents, succinimidyl esters and lipophilic dyes (*see* **Notes 2–4**). CTV is a succinimidyl ester.

2.4.1. CTV is supplied as a lyophilized powder (stored at  $-20$  °C till the manufacturer's expiration date) that is reconstituted in 20  $\mu$ l of DMSO to a stock concentration of 5 mM. Working concentrations range between 1 and 10  $\mu$ M and should be determined empirically for each cell type. Aliquots of reconstituted dye can be stored at  $-20$  °C and freeze-thawed once. It is excited by the 405 nm laser and emits at 450 nm (Fig. 1, lower panel).

2.4.2. The exact *activation reagents* required depend on the immune cell type being studied for ACD. All culture, activation and staining/washing steps are done in 96 well round bottom plate or 24-well flat-bottomed plates (various suppliers).

#### 2.4.3. F5 transgenic T cell activation reagents:

- NP68 peptide (synthesized in house): The stimulatory peptide for the F5 receptor.
- Anti-CD3 antibody (clone 145-2C11): A generic T cell activator.
- Anti-CD28 antibody: A co-stimulatory signal.
- Recombinant ICAM-1: Thought to be essential for driving ACD.

#### 2.4.4. B cell activation reagents:

- CD40 ligand. Mimics effect of a helper T cell signal.

- Anti-IgM antibody: Stimulated through the B cell receptor.
  - Interleukin-4: T cell-derived cytokine.
- 2.5. *Antibodies against intracellular and extracellular targets* should be chosen depending on the molecules/cell cycle phases under investigation. For T cell studies CD69 or Ki67 are used to identify cells that have been triggered to divide. This allows one to determine the best harvest window in terms of the greatest number of cycling cells [12]. Antibodies should be directly conjugated when possible with fluorochromes compatible with the overall panel and spectral properties of the IFC instrument. Species, clones, and cross-reactivity should also be considered (*see Note 5*). For particular markers/antigens we strongly recommend using the indicated fluorochromes (shown in bold type). For markers where no particular fluorochrome is recommended, one can be selected that fits into a free channel on the IFC system used (*see Fig. 1*). Examples of some marker/fluorochrome combinations we have used are:
- 2.5.1. Anti-Ki67: Used for measuring cell cycle entry.
  - 2.5.2. Anti-CD69 (clone H1.2F3): Denotes commitment to proliferation.
  - 2.5.3. Anti-MPM2 (clone MPM2), for example FITC or Cy5 conjugated (*see Note 5*): Identifies all stages of mitotic cells.
  - 2.5.4. Anti-PKC zeta goat anti rabbit (cat no SC-216-G, Santa Cruz, USA): Strongly implicated as an asymmetrically inherited fate marker.
  - 2.5.5. Donkey anti-goat PE (CH3) or AlexaFluor-647, (CH11): To detect the unconjugated PKC zeta goat antibody (*see Note 5*).
  - 2.5.6. Anti-CD8 (clone 53-6.7): A possible marker for T cell ACD.
  - 2.5.7. Anti-CD25 (clone PC61.5): A possible marker for T and B cell ACD.
  - 2.5.8. Anti-CD86 (clone GL1): A possible marker for B cell ACD.
  - 2.5.9. Anti-IA/IE (clone M5/114.15.2): A possible marker for B cell ACD.
- 2.6. FC-block reagent (anti CD16/32 clone 2.4G2): Used to block nonspecific binding of antibodies via their Fc regions.
- 2.7. *Near-infrared live/dead (NIRLD) fixable dye* (CH12): Note that this dye is not compatible with an IS100, but can be used on a single-camera ISx system in CH6. BF illumination

can also feature in this channel as NIRLD signal from dead cells will cause “white out” in the BF image and dead cells can be gated out using the gradient RMS feature for focus along with defocused cells.

- 2.8. Fixatives: Will depend on the overall antibody panel used and purpose of experiment (*see Note 6*).
  - 2.8.1. 70 % Ethanol (EtOH): Made up in deionized water (DI H<sub>2</sub>O) and stored at 4 °C.
  - 2.8.2. 4 % Formaldehyde (FA) stock: Diluted in PBS (*see Note 6*).
- 2.9. *Propidium iodide/7-AAD*: Both are available from several commercial sources. Stocks are made up at 50 µg/ml in DI-H<sub>2</sub>O.
- 2.10. *Siliconized 1.5 ml microfuge tubes* or protein “low-bind” version.
- 2.11. *Copies of IDEAS (Amnis), FlowJo (Treestar Inc.) or equivalent FCS-based software analysis package*: Image J (open-source image analysis package, NIH, USA). This protocol assumes a good working knowledge of these software packages. For IDEAS, users should understand compensation, masking, and feature creation. Users should also know how to export feature values and raw images for analysis in third party software. More information can be found in the respective user guides.

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## 3 Methods

### 3.1 CTV Labeling

#### 3.1.1 Labeling Primary T and B Cells with CTV

Cells are washed once in complete culture media and resuspended for cell counting. Centrifugation conditions are 1200 rpm/300 × *g* for 3–5 min followed by the removal of all supernatant (hereby referred to in this protocol as “washing cells”). Cell viability should also be determined using a dye exclusion method such as trypan blue.

CTV staining solution is made up in protein-free sterile PBS and pre-warmed at 37 °C prior to use. Concentrations for primary T and B cells should be determined empirically based on viability and uniformity of the CTV signal [19]. Labelling solutions should be made up at 2× concentrations. Typical final concentration used for T and B cell labeling is 2.5 µM.

Cells should be spun down and the supernatant carefully aspirated away as any residual protein can compete out the intracellular CTV labeling. The cell pellet should be resuspended in one volume of protein-free PBS so that once the 2× labeling solution is added at an equal volume, the final cell concentration should range between 1 and 3 × 10<sup>6</sup>/ml. The cells should then be incubated at 37 °C for 10 for 5 min (B cells) or 10 min (T cells).

After incubation, neat FBS should be added to the labeling solution to a final concentration of 10 % and incubated for a minimum of 5 min at room temperature. This will terminate intracellular labeling by binding any remaining free dye from the solution.

Cells should then be washed once in full media and resuspended for counting and viability assessment. An aliquot of cells should also be analyzed by CFC in order to determine the intensity and uniformity of CTV labeling as this affects division peak resolution (*see* **Notes 2** and **7**). Cells can then be adjusted to the appropriate density for subsequent culture and activation.

### 3.1.2 Labeling Cell Lines

Cell lines are labeled with CTV as described above with no further modifications.

In our hands, CTV successfully labels a number of cell lines (e.g., Jurkat/A549); however the labeled width always exceeds the limits for division peak resolution due to cell-intrinsic heterogeneity (*see* **Note 7**). This requires cells to be sorted prior to culture in order to narrow the input width sufficiently to resolve division peaks. This method is beyond the scope of this protocol and is described in full detail elsewhere [19].

## 3.2 Culture and Activation Conditions

### 3.2.1 T Cells

An example of the in vitro T cell activation protocol from our lab has been described elsewhere [12]. However, briefly CTV-labeled T cells are seeded in 24-well plates at a density of  $1 \times 10^6$ /ml in the presence of various activators, including peptide loaded dendritic cells or anti-CD3 (145-2C11, plate bound at 10  $\mu$ g/ml or soluble at 0.5  $\mu$ g/ml) with or without plate bound CD28 and/or recombinant ICAM-1 (both at 10  $\mu$ g/ml). The cells are harvested at the point of cell cycle commitment based on Ki-67/CD69 expression in the CTV-undivided population (*see* **Note 8**). Typically this tends to be between 32 and 36 h. for MHC-I-restricted F5 CD8 T cells [12].

### 3.2.2 B Cells

CTV-labeled B cells are seeded in 24 or 96 well-flat bottom plates at a density of  $1 \times 10^6$  cells/ml. To stimulate B cell proliferation we add CD40L at concentrations ranging from 1 to 0.001  $\mu$ g/ml or anti-IgM at concentrations ranging from 10 to 0.001  $\mu$ g/ml. In all cases, B cell culture media must be supplemented with 5 ng/ml IL-4. In our system, B cell proliferation begins 48 h after stimulation with an optimal harvest time of 72 h.

### 3.2.3 Culturing Cell Lines for Chemotherapeutic Drug Assays

CTV-labeled cell lines, pre-sorted to optimal input width for division peak resolution, are placed back into culture at a density of  $0.5 \times 10^6$  cells/ml in complete media with a total minimum cell number of  $1 \times 10^6$  cells (2 ml) per well per analysis time point.

An aliquot of post-sorted cells ( $1 \times 10^6$  cells) are fixed and stained to determine the input cell cycle distribution. Cells are allowed to recover post-sorting for a minimum of 24 h before addition of any cell cycle modulating compounds.

At the point of drug addition, a time zero sample is taken ( $1 \times 10^6$  cells) and fixed under appropriate conditions as described later in the protocol. As a control, cells are also incubated in the absence of drug for the full culture period. Cells can then be cultured for defined periods in the presence or absence of compound.

To model the effects of drug metabolism and removal, one can also investigate the effects of washing the drug away and re-culturing the cells to determine long-term irreversible effects on proliferation to model the effects of drug clearance. The ability to track cells over multiple cell divisions using CTV (~6 rounds) makes such long-term tracking a viable option compared to other temporal methods. As a control at this stage, a sample of cells is taken and fixed at the point of drug removal. A further control is set up where the presence of drug is maintained throughout the culture period.

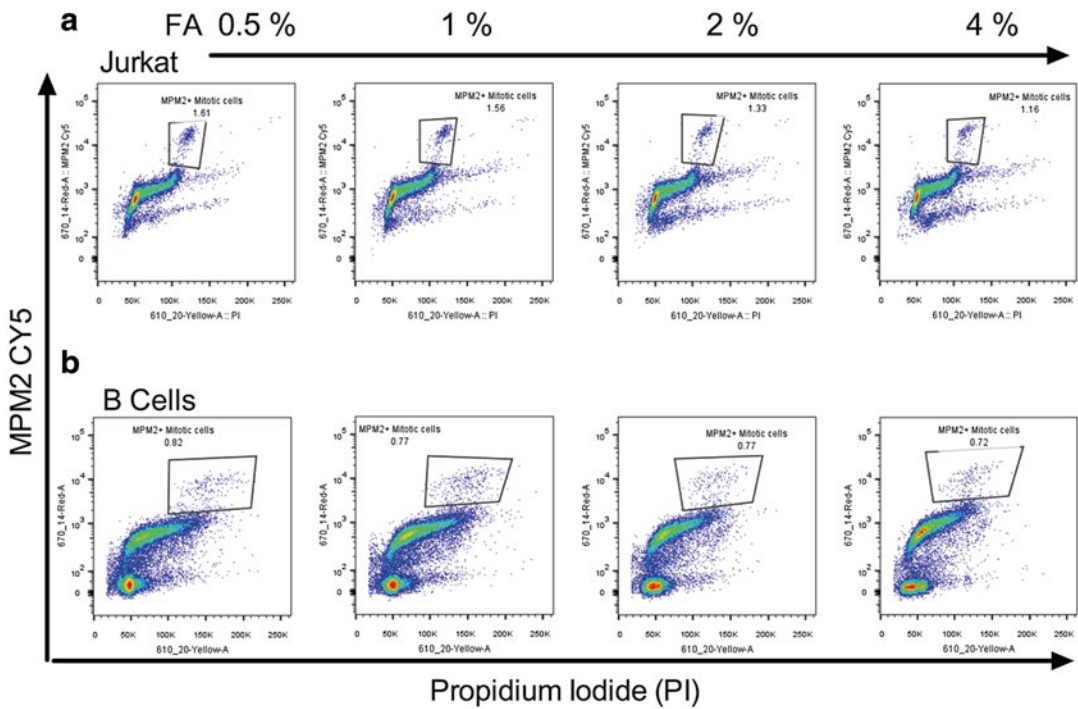
### **3.3 Live/Dead Fixable Dyes (NIRLD)**

Harvest cells from culture and wash  $\times 1$  in wash buffer. Add 2  $\mu\text{l}$  of LD-NIR per ml and incubate at RT for 10 min. Wash cells again then proceed to extracellular staining.

### **3.4 Extracellular Antibody Labeling**

1. This stage is only required if surface markers are to be investigated. Furthermore, if the surface epitopes are unaffected by fixative treatment, then all antibodies (extra- and intracellular) can be added post-fixation/permeabilization. All antibodies and dyes should be optimally titrated for use on an IFC system paying particular attention to the relative brightness of all fluorochromes excited by the same laser (*see Note 9*).
2. Make up the cocktail of extracellular antibodies at the pre-optimized concentrations in stain/wash buffer. Add 100  $\mu\text{l}$  per well to stain  $1 \times 10^6$  cells. If you are using tandem dyes, be sure to check that they remain stable after fixation. Also pay attention to the fact that tandem dyes will be prone to cross laser excitation (*see Note 9*).
3. For staining, cells should be seeded in a round-bottomed 96-well plate at a density of  $1 \times 10^6$  cells/ml. Often we stain 2–3 million cells in total per sample. This will require setting up multiple wells per sample to be pooled at the end of staining.
4. If required, incubate cells with anti-FC block made up in stain/wash buffer for 10 min, then wash. After removing the supernatant, briefly press plate bottom onto a vortex mixer to break up the cell pellets. This can be done after every wash step.
5. Add 100  $\mu\text{l}$  of antibody cocktail per well and incubate cells in the dark at RT for at least 1 h. Wash cells twice in stain/wash buffer and incubate with secondary labelled antibodies if required.





**Fig. 2** Identifying mitotic cells by MPM2 staining. **(a)** Jurkat cells and **(b)** primary mouse B cells were fixed with the indicated concentrations of FA as outlined in Subheading 3 and stained with PI (x-axis) and MPM2 (y-axis)

6. Single stained controls for each fluorochrome in the panel should be prepared and processed exactly as experimental samples. Allow for the inclusion of single stains against intracellular targets by preparing these wells in advance. Also set up further control wells containing unlabelled cells at exactly the same cell density as experimental samples. These will serve as the PI compensation single cell control when the dye is added to the samples at the end of the staining process.

### 3.5 Fixation (See Note 6)

#### 3.5.1 70 % EtOH Fixation

If you are only measuring cell cycle distribution without extensive antigenic staining then 70 % EtOH fixation is optimal as it gives the best DNA profiles. It is also compatible with MPM2 staining for mitotic phases [19].

For fixation, ice-cold 70 % EtOH is added to the pellet and then samples are incubated for a minimum of 1 h at RT. EtOH fixed cells can be kept in fixative for 24–48 h without detrimental effects on the CTV peak resolution.

#### 3.5.2 Formaldehyde Fixation

ACD analysis will require staining for antigens in addition to MPM2; therefore EtOH will likely destroy the epitopes necessitating a switch to FA. We have found that a lower concentration of formaldehyde (0.5 %) work best for DNA and MPM2 staining resolution in cell lines (Fig. 2a) and primary murine B cells (Fig. 2b).

The cell pellet is first resuspended in one volume of PBS to eliminate clumping. An equal volume of 2× FA solution is then added to a final desired fixative concentration (0.5 %). Incubation should be done at RT for a minimum of 1 h. Cells can be washed out of fixative and stored in stain/wash buffer for up to 24–48 h without loss of CTV peak resolution.

### 3.6 Intracellular Antibody Labeling

1. Cells are first permeabilized in 100 µl of permeabilization buffer for a maximum of 5 min, after which 100 µl of wash buffer is added prior to centrifugation. Next add the cocktail of intracellular antibodies made up at appropriate dilutions in wash/stain buffer and incubate at RT in the dark for minimum 1 h. At the same time, stain for any single-stain compensation samples that require antibodies against intracellular antigens.
2. Wash cells a minimum of two times. If required, incubate with fluorescently labeled secondary antibodies made up in wash/stain buffer for a minimum of 45 min in the dark at RT, after which wash cells once.
3. The final volume for sample resuspension should not exceed 60 µl (*see Note 10*). Pool multiple wells if needed by adding 60 µl of stain/wash buffer to the first well and transfer to subsequent wells until there is approximately  $2\text{--}3 \times 10^6$  cells in the 60 µl volume. Transfer to a 1.5 ml siliconized, low-bind microfuge tubes.

### 3.7 DNA Dye Staining

1. PI concentration should be pre-titrated to avoid camera saturation at a given laser power (*see Note 9*) while still giving well-defined DNA profile based on G1 CVs (Fig. 3a).
2. Add PI to a final concentration of 1 µg/ml per 2–3 million cells and incubate samples for 10–20 min at RT to ensure saturation of the DNA. If cells are fixed in FA, then we use the same concentration of PI solution containing 0.1 % triton X-100 (*see Note 6*).

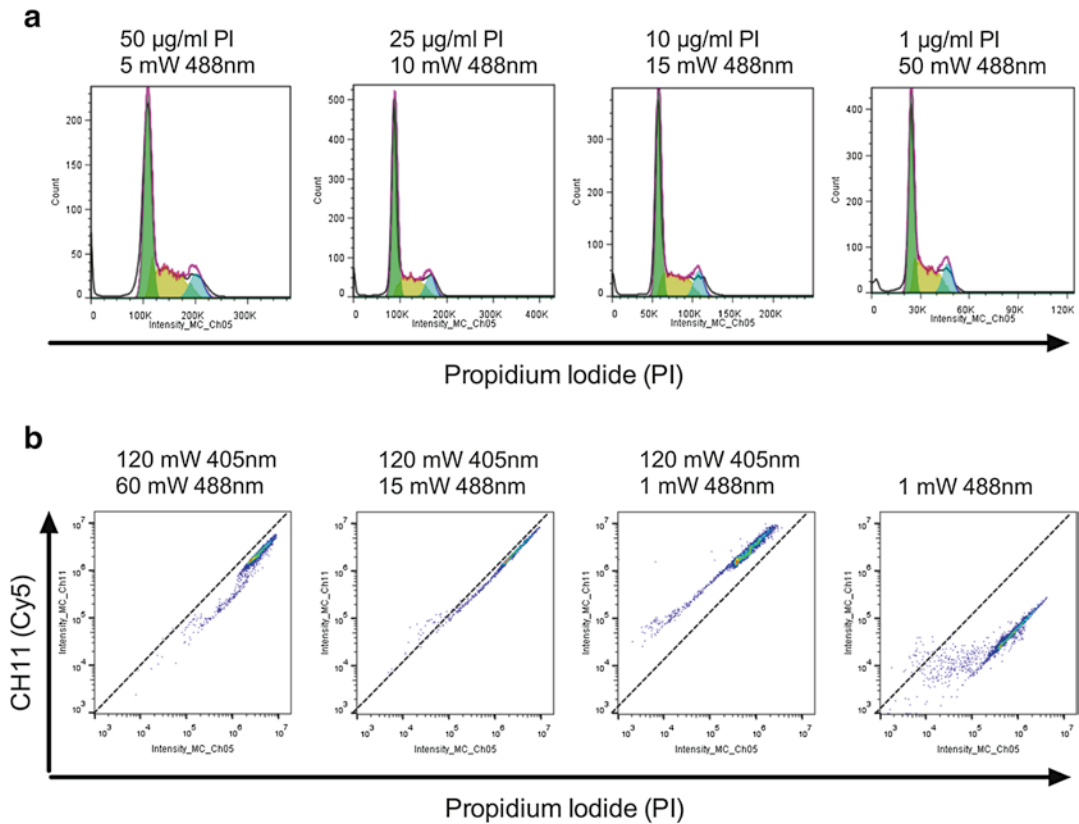
### 3.8 Sample Acquisition (12 Channel Systems, FS/ISx)

#### 3.8.1 Setting Excitation Laser Powers

Collect bright-field (BF) illumination in CH1 and CH9. As side-scatter information is not required the 758 nm laser can be turned off.

Switch on all required excitation lasers at least 20 min before acquisition and set the powers based on the raw max pixel feature for each individual fluorescence channel. Aim to achieve the best possible total fluorescence signal (akin to pulse area on CFC instruments) while avoiding saturation of the camera based on Raw Max Pixel values (12-bit CCD, scale range from 0 to 4096, with 4096 denoting saturation akin to pulse height on a CFC system).

For primary cell acquisition on an ISx, select 60× magnification with the high-sensitivity fluidics mode. Cell lines can be analyzed effectively at 20× or 40×.



**Fig. 3** Optimising the PI concentration for IFC analysis **(a)** Fixed Jurkat cells were stained with decreasing levels of PI and acquired at increasing 488 nm laser powers as indicated. The PI fluorescence was analyzed by exporting as an FSC file and using the modeling option in FlowJo [33]. In all cases the G1 CV was <9 and the % of cells modelled in each phase was comparable (data not shown). **(b)** Fixed Jurkat cells were labeled with 50 µg/ml PI and acquired on a 12-channel IFC system using the indicated 488/405 nm laser powers. The 45° lines drawn through the origin for each plot denote the limits of acceptable spill over whereby PI fluoresces becomes brighter in CH11 than CH5 and would therefore require over 100 % compensation (not possible by IFC)

As well as guarding against CCD channel saturation, look for issues caused by cross laser excitation of particular dyes in the panel. For example PI is excited maximally by the 488 nm but also 20 % by the 405 nm lasers (*see Note 9*). Staining with high PI concentrations will require setting a low 488 nm laser power to avoid signal saturation. If the 405 nm laser is set relatively high in order to excite CTV or other dyes, then it is possible PI emission will be maximal in CH11 rather than CH5 (Fig. 3b). If this happens, then the data is effectively ruined so this is why titration of PI is essential when working with a multicolor panel (Fig. 3a, b).

### 3.8.2 Setting Limits of Event Acquisition

Aim for a final analysis population of >100 cells and base the number to acquire on the frequency of these event within the total population. For example to obtain 200 telophasic cells from the

undivided population at a target frequency of 0.001 %, aim to collect 200,000 live cells. Concatenate samples if needed post acquisition using IDEAS.

### 3.8.3 *Sample Acquisition Order*

First run a sample stained with all fluorochromes apart from the DNA dye. This sample should ideally have the brightest staining levels of all markers. Set the laser powers as outlined above and do not change them from this point onwards.

Acquire single-stained samples individually with bright-field illumination and the 758 nm scatter laser turned off.

Lastly, add PI to the unstained sample at the pre-optimized concentration/incubation time for the selected 488 nm laser power. Acquire this as the PI compensation control (*see Note 9*).

Add the same concentration of PI to the experimental samples (e.g., 1 µg/ml, 15 min) and collect the data.

If any samples not requiring PI need to be run after a PI-stained sample, clean the instrument by loading a tube of bleach-based solution followed by DI-H<sub>2</sub>O.

## 3.9 *Data Analysis*

### 3.9.1 *Multispectral Compensation*

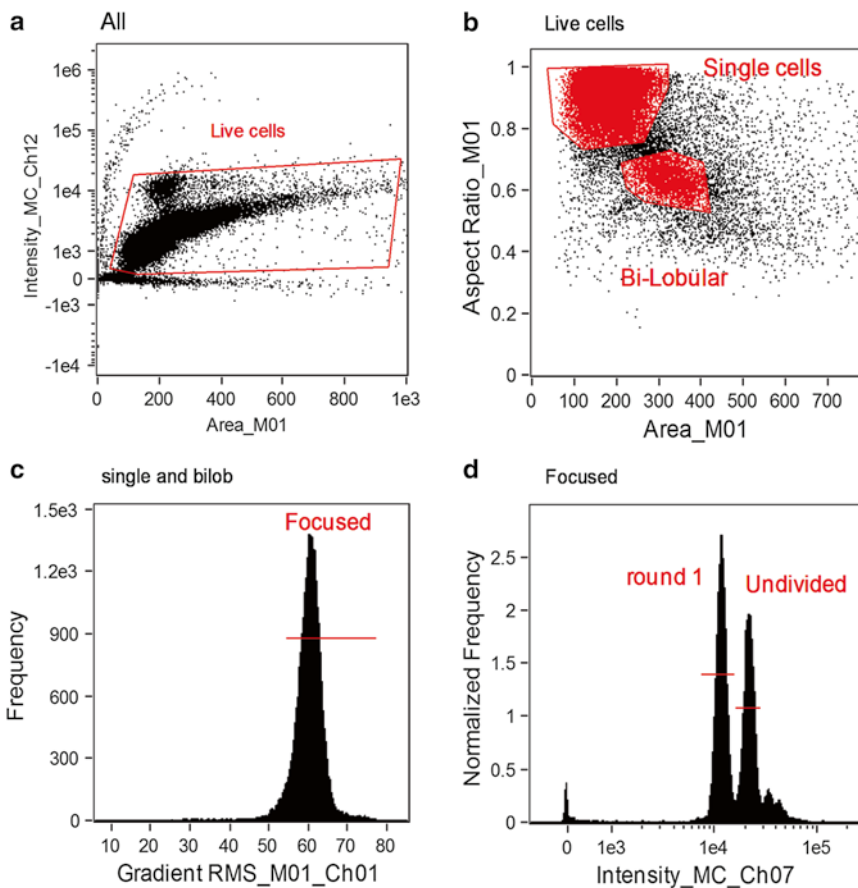
Perform compensation using the embedded wizard in the IDEAS software, the principles of which have been extensively described elsewhere [20, 16, 21]. Briefly, the .rif files for each single fluorochrome stain are selected and loaded into the wizard. Only select the imaging channels known to contain the peak emissions signals of the fluorochromes in your panel. IDEAS then calculates the coefficient of the fluorescence relationship for each dye in the specific channel versus each cross talk channel. Coefficients are presented in a 12 × 12 matrix format. Manually inspect each distribution to identify debris and autofluorescence that would skew the best fit line and affect compensation. If required, refine the compensation populations to eliminate any such skewing. Finally, validate the matrix values on the imagery for each fluorochrome channel.

### 3.9.2 *Identifying Live, Focused, Single/Bilobular Cells*

Do not use any of the embedded analysis wizards in IDEAS software. However the use of the building blocks option for predefined plots is very useful. Initial measurements and features are derived from the pre-calculated channel masks (e.g., defaults masks M0<sub>x</sub>, where <sub>x</sub> denotes channel number, or MC that denotes the sum of all masks):

First, identify live cells based on NIRLD total fluorescence levels (MC, CH12) and the area of the BF image (Fig. 4a).

Next use the area versus aspect ratio of BF image creating two gates, one for single cells and one for bilobular cells. Next, construct an “OR” Boolean population of single and bilobular cells for subsequent analysis so as to include telophasic cells (Fig. 4b)



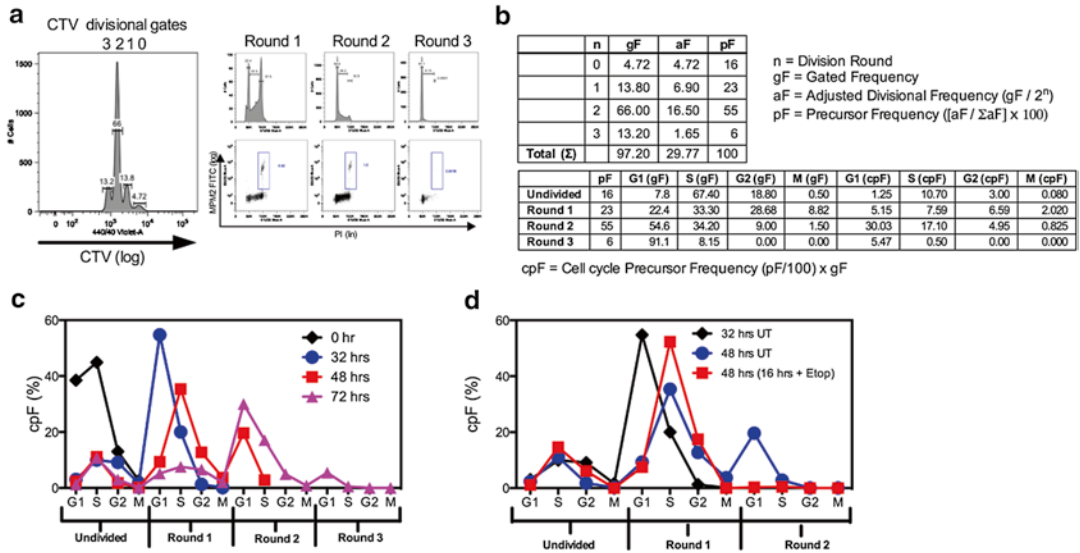
**Fig. 4** Initial gating strategy for IFC-based cell cycle/ACD analysis. **(a)** Live cells are identified based on NIRLD fluorescence in CH12 ( $y$ -axis) and BF area ( $x$ -axis). **(b)** Single and bilobular cells are identified based on the area ( $x$ -axis) and aspect ratio ( $y$  axis) of the BF image. **(c)** Focused cells are selected by gating on the gradient RMS measurement of the BF image. Focused cells are gated as shown ( $>50$ ). **(d)** CTV fluorescence in CH7 is plotted as a histogram and gated to identify different proliferative generations

Next identify focused cells based on the gradient RMS of the BF image (M01, CH1) and gate accordingly (Fig. 4c).

Manually inspect the CTV fluorescence histogram (MC, CH07) and set region markers to identify individual division rounds (Fig. 4d).

### 3.9.3 Analysis of Division History and Cell Cycle Distribution in the Presence of Chemotherapeutic Compounds (See Notes 11 and 12)

From the live, single/bilobular, focused population begin by using the CTV, PI and MPM2 distributions to gate and record the frequencies of the major cell cycle stages from within each division round (Fig. 5a). Adjust the frequency of cells within each CTV peak by  $2^n$ , where  $n$ =the division round to account for the doubling effect every time an input cell has divided. It is then possible to derive the true frequency of the input population within each division round by dividing the adjusted frequencies by the sum of all adjusted frequencies (Fig. 5b, upper table).



**Fig. 5** The workflow for calculating the cell cycle precursor frequency (cpF) of an asynchronously dividing population stained with CTV, PI, and MPM2. **(a)** Region gates are set on the CTV histogram to identify division rounds. PI/MPM2 fluorescence is used to derive the major cell cycle stages within each peak through gating. **(b)** The gated frequencies within each CTV peak are corrected as outlined to account for the input population expanding by  $2n$ , where  $n$  is the division round. These adjusted frequencies represent the true frequency of the input population within each division round. We can then use the cell cycle stage frequencies to determine the frequency of the input population within each phase (cpF). **(c)** The cpF values ( $y$ -axis) can be plotted as a function of each temporally distinct cell cycle phases ( $x$ -axis) over an increasing culture period. **(d)** Using this method, one can add various chemotherapeutic drugs (the s-phase blocker etoposide in this case) for a short time window and conclusively determine what effect they exert on the temporal evolution of the labeled population

Take the adjusted frequencies for each division round and break those down by cell cycle phase using the gated frequencies from the PI versus MPM2 plots (Fig. 5b, lower table).

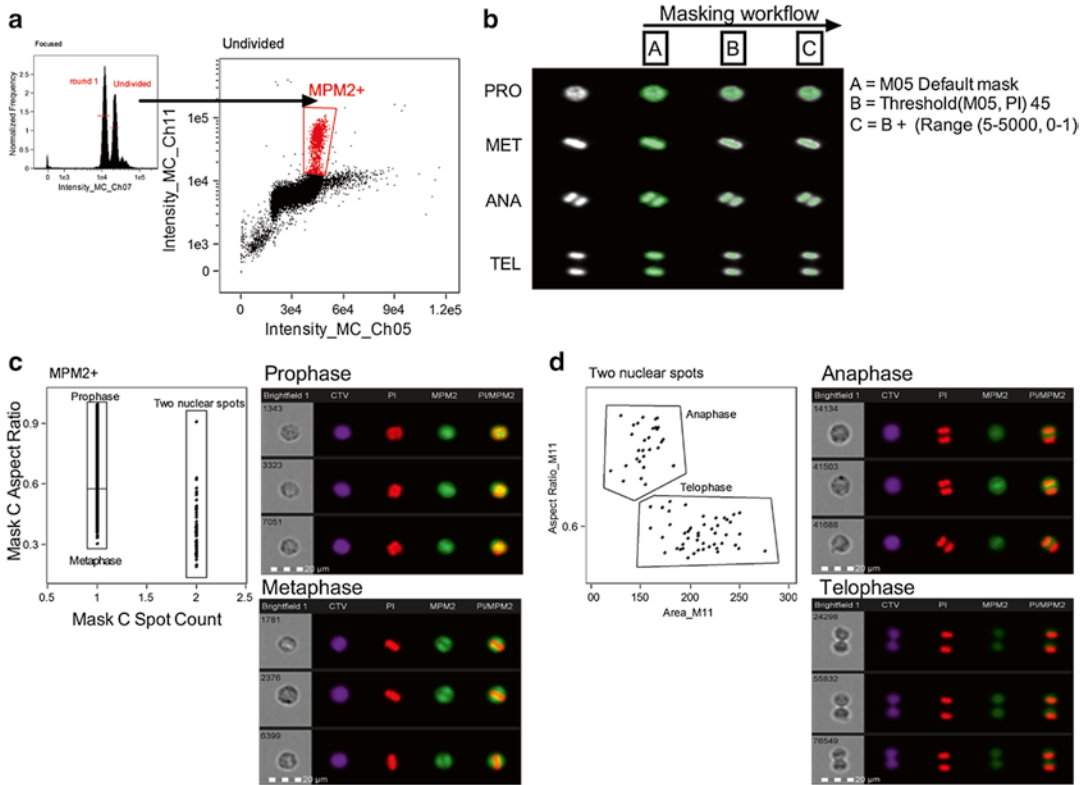
This then allows one to plot the temporal evolution of the input population as it moves through and becomes distributed over multiple cell cycles (Fig. 5c).

If one harvests, stains and analyses cells prior to the addition of chemotherapeutic agents it is possible to establish how the cells are distributed prior to exposure. One can then incubate cells in the presence and absence of drug (Fig. 5d) and determine exactly where and when the drug effect occurs.

By then using imagery to subdivide the mitotic phase (*see* next section), one can incorporate the frequencies of prophase, metaphase, anaphase, and telophase cells into the analysis framework.

3.9.4 Identifying MPM2 Positive Mitotic Cells with Defined Division Histories

The most reproducible way to identify and subdivide mitotic cells is to stain for a unique mitotic antigen (MPM2) and then use custom masking and morphometric/intensity-based features. Relying solely on spatial parameters without unique fluorescence, as employed by



**Fig. 6** Using IFC-derived image data to subdivide mitosis. **(a)** Cells undergoing first mitosis can be identified based on CTV (CH7), PI (CH5), and MPM2 (CH11) intensity as shown. **(b)** In order to best resolve the different mitotic phases from within the MPM2+ gate based on the PI-stained nuclear image, the default channel mask must be adapted as shown. Note that the default mask does not distinguish the two nuclear poles in anaphase or telophase cells without adaptation. The masking workflow is shown using the language of IDEAS for audit and reproduction purposes. **(c)** A bivariate plot of the spot count (*x*-axis) and aspect ratio (*y*-axis) of mask **c**. Gates are set to identify prophase and metaphase cells from within the 1-spot population. Multispectral images are shown as examples at 20 $\times$  magnification. **(d)** The two spot population is further refined using a bi-variate plot of the area (*x*-axis) and aspect ratio (*y*-axis) of the MPM2 image (M11). Gates are set to distinguish anaphase from telophase cells. Multispectral images are shown as examples at 20 $\times$  magnification

the IDEAS cell cycle wizard, leads to a high degree of misclassification (data not shown).

Firstly, create bivariate plots of PI total intensity (CH05, MC) versus MPM2 total intensity (CH11 Cy5, or CH2 FITC, MC) for each division round. Set a gate to include all MPM2+ events that have a 4N DNA content based on PI intensity (Fig. 6a). The MPM2 phospho-epitope, unlike pH 3, is maintained in telophasic cells making it easier to resolve them from debris and doublets rather than relying on complex masking [16].

Use a simple set of masking adaptations based on the default PI channel mask (M05). The masking adaptation workflow is shown in Fig. 6b (*see Note 13*). The aim is to create a mask that

better represents the morphological differences in the nuclei of different mitotic phases [21].

Calculate the aspect ratio and spot count features from the final mask. Create a bivariate plot of spot count ( $x$ ) versus aspect ratio ( $y$ ) and gate to subdivide 1 spot, high aspect ratio cells as prophase, 1 spot mid-aspect ratio events as metaphase, and all two spot events (Fig. 6c).

The two spot events are plotted on a bivariate graph of MPM2 (CH11, M11) area versus aspect ratio. Anaphase cells have low area and high aspect ratio, whereas telophasic cells (*see Note 14*) have an increased area and a mid to low aspect ratio (Fig. 6d).

**3.9.5 Measuring Signal Symmetry/Asymmetry**  
(Only Required for Analysis of ACD)

For prophase cells that have no clear plane of division we use IDEAS to calculate the delta between the BF channel centroid and the intensity-weighted centroid of the fluorescence channel of our marker we are investigating (Fig. 7a). In both cases we use the MC as the input mask for this feature. It is essential that the intensity weighted centroid is used otherwise there is a potential to misclassify polarity depending on how the overall signal intensity is distributed (Fig. 7b). In this case, fluorescently labeled antigen-coated beads can be distributed in several possible configurations and the feature used to calculate signal polarity must be robust to the possibility that the beads may be globally distributed, but the relative intensity of the foci may still be polarized [17].

In metaphase, anaphase, and telophase populations where the plane of cytokinesis can be clearly defined (Fig. 7c) we export the 16-bit raw tiff channel images for the PI (CH5), CTV (CH7), and fate determinant (e.g., CD8 and PKC $\zeta$ ) fluorescence channels. This is done from the final gated populations through IDEAS export option.

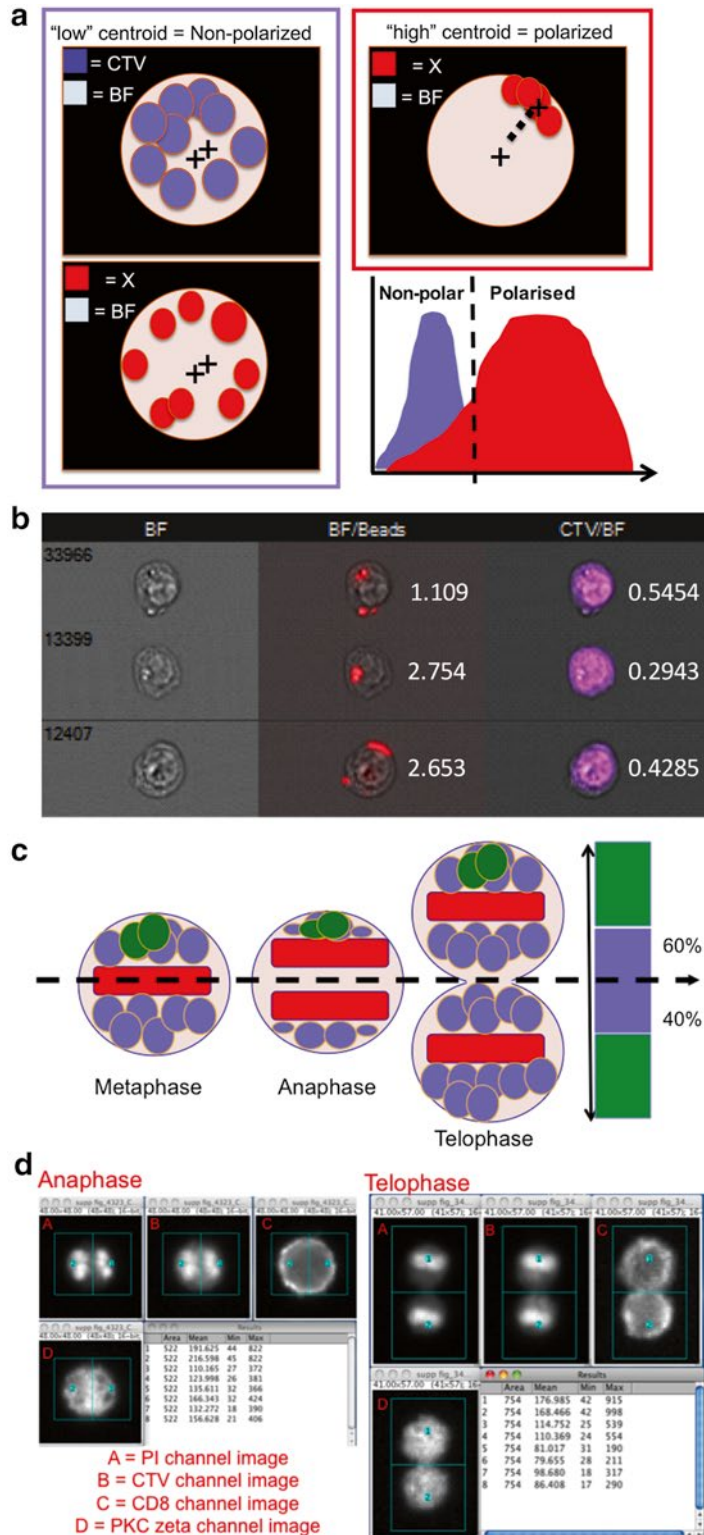
These images can then be analyzed rapidly by software such as Image J either manually or, if cell numbers are impractical, using an automated script. In all cases, the putative daughter poles are segmented across the plane of cytokinesis using the nuclear PI image as a guide (Fig. 7d).

One can use the fluorescent distribution of CTV and PI as an internal control for symmetry and polar focus. This has been described as a “Morphometrically Relevant Biological” (MRB) control (*see Notes 15 and 16*). In prophase cells, the MRB control is the intensity weighted delta centroid between CTV and BF images.

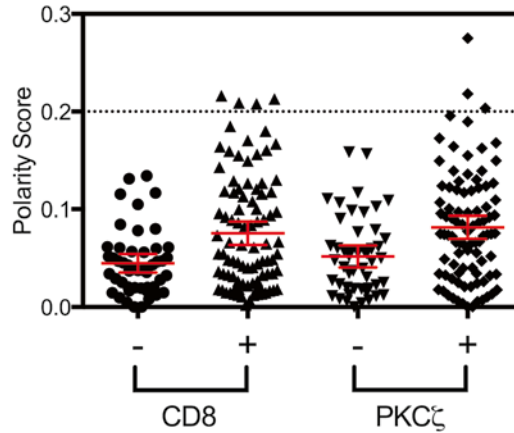
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**Fig. 7** (continued) the respective centroid values shown for BF vs. beads and BF vs. CTV signals. **(c)** A cartoon depicting the use of CTV fluorescence distribution across the plane of division in metaphase, anaphase and telophasic cells to set the limits of symmetrical inheritance. The green signal exceeds the 40–60 % limit so is considered asymmetric **(d)** An example of using ImageJ to segment putative daughter poles in anaphase and telophasic cells to then measure the pixel intensities within these areas for all the indicated fluorescent channels. Images are shown at 40 $\times$  magnification





**Fig. 7** Quantifying polarity and asymmetry using IFC-derived mitotic imagery. **(a)** A cartoon denoting how the intensity-weighted delta centroid measurement can be used to determine the limits of non-polarized and polarized fluorescence distributions. **(b)** An example of using these measurements on primary CTV-labeled B cells loaded with fluorescent antigen-labeled beads [17]. Multispectral images are shown at 40 $\times$  magnification with



**Fig. 8** Plotting the polarity index of CD8 and PKC $\zeta$  in antigen-activated F5 CD8 T cells entering first division. (a) F5 CD8 T cells were labeled with CTV and activated with NP68 peptide under ACD-inducing conditions in the absence (–) or presence (+) of cytochalasin B for the last 4 h of culture. Late-stage mitotic cells from within the undivided CTV peak were then analyzed as described in Subheading 3 and the polarity index was calculated. Values >0.2 are considered to be polarized based on our MRB control. Data are adapted from Hawkins et al. [12]

This is used to set the limits for symmetrical signal distribution (Fig. 6a). In other cell types with a clear divisional plane, total protein is normally distributed within the 40–60 % limits based on CTV intensity and DNA distributed between 45 and 55 % based on PI intensity.

The number of cells analysed using our IFC-based method means that one can plot data as population distributions and use the appropriate statistical tests. Data can be expressed as the percentage polarized or median polarization score for prophase cells [17]. Or for cells with a clear plane of division one can construct a polarity index as described previously [12, 19]. The equation for polarity index calculation is shown below (Eq. 1):

$$\text{PolarityIndex} = \frac{\sqrt{(\% \text{in pole} - 50)^2}}{50} \quad (1)$$

A score >0.2 was considered to be polarized or asymmetric with respect to signal inheritance over the cytokinetic plane. Example data from F5 CD8 T cells stained with our panel and activated under conditions considered to drive ACD is shown in Fig. 8.

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## 4 Notes

1. We use a Becton Dickinson LSRFortessa (BD, USA) system with 405 nm, 488 nm, 561 nm, and 633 nm laser lines and a standard filter configuration.
2. We have obtained the optimal division peak resolution using the succinimidyl ester CellTrace Violet™ (CTV). It is non-cytotoxic, retained within the cells, inherited in a symmetrical fashion by daughter cells during cytokinesis and fluorescently stable after fixation. The excitation and emission properties of the dye make it spectrally compatible with both CFC and IFC systems in combination with a range of other fluorochromes. Moreover it can be used to reliably define the limits of symmetrical protein inheritance as an internal control [19, 16, 12, 17].
3. Lipophilic dyes such as PKH26 tend to yield poorly resolved division peaks compared to most succinimidyl esterase dyes. This may be due to mechanisms such as membrane exchange [22] that influence the relationship between fluorescent intensity and how many times a cell has divided. If for example a cell that has divided twice takes up some dye from an adjacent cell, it may now have an intensity that makes it appear as though it has not divided at all. Conversely, a cell that has not divided may lose some dye and appear as though it has undergone division. For studies of ACD where the division history of cell must be accurately determined, such sources of error must be eliminated where possible.
4. Of the succinimidyl esterase-based dyes currently available, we have found eFluor proliferation dye 670™ highly unsuitable for division tracking as firstly it transfers heavily to bystander cells and secondly it labels a discrete number of cellular structures rather than all intracellular protein. This means that due to a stochastic process [23], the fluorescent signal is not always inherited within symmetrical limits rendering it unsuitable for accurately tracking cell proliferation [19].
5. As well as cross reactivity between primary and secondary antibodies, one should also check that mouse derived clones do not bind non-specifically to mouse cells (B and T). In experiments with B cells, special care must be taken regarding the usage of secondary antibodies because as B cells proliferate they start to express different immunoglobulin (Ig) isotypes on their surface, such as IgG1. To avoid issues with cross reactivity, it is strongly suggested to use directly conjugated antibodies, particularly for MPM2, which is a mouse IgG1. MPM2 can be obtained directly conjugated to both FITC and Cy5 (*see* Subheading 2). Another alternative is to select primary unconjugated antibodies that are not raised in mouse thereby avoiding the need for anti-mouse secondary labeling reagents.

6. The choice of fixative will depend on the application of our method. If it is purely for analyzing cell cycle using PI and MPM2 then 70 % EtOH works best as it dehydrates the cells allowing easy access to the nucleus for DNA-binding dyes without affecting the MPM2 epitopes. However, if more complex antigenic staining is required, often EtOH fixation will destroy binding epitopes meaning that FA must be used. FA fixed cells tend not to give such well resolved DNA histograms as the cross-linking affects dye access to the nucleus. This can be improved somewhat by including 0.1 % triton-X 100 when incubating with the dye. When making up FA fixative solutions, one should start from liquid state rather than paraformaldehyde powder. The reason for this is that the actual fixative is monomeric formaldehyde, or methyl-hydrate. Methyl hydrate formation is most rapid and stable when formaldehyde is diluted in neutral buffers such as PBS. As such we always make up our FA solution in PBS and leave it for at least 2 weeks at 4 °C prior to use. If cells are not properly fixed prior to permeabilization, then the cells will simply fall apart. Fixed cells should also have reduced forward and side scatter signals compared to unfixed cells. We routinely use these metrics to determine the activity of a fixative solution.
7. The ability to resolve division peaks using fluorescent dye dilution is determined by several interrelated factors. Firstly, cytometer performance must be optimal in terms of laser excitation and fluorescent detection so as to minimise the contribution of machine errors to the measurement. Secondly, labeling conditions must be optimised to ensure tight, uniform populations. However, heterogeneous cell types (cell lines, lymphocyte blasts) have variable protein content and will always label with a broad distribution even under fully optimized conditions. These cell types require cell sorting to narrow the input population below a certain threshold for peak resolution [19].
8. The harvest time is very important for the analysis of rare events such as telophasic cells. These targets can be less than 0.01 % of the total population. Typically we aim to collect between 100 and 500 cells for the final measurement as this allows us to use population statistics to describe the biological differences we may see. We want to avoid any inhibitors of cytokinesis or block and release protocols as we have shown that cytochalasin treatments may alter the natural cell state and induce artifactual asymmetry [12]. Therefore we need some idea of the window when cells are entering first division by using markers that denote entry into G1 such as Ki67 or CD69. We suggest that CTV-labelled primary cells are cultured under activating conditions and harvested at different time

points, fixed, and stained for Ki67/CD69 to denote activation/cell cycle commitment. This can be analysed by CFC and does not require an IFC system.

9. One of the biggest challenges with acquiring samples on an IFC system is the lack of control over individual camera channels. Unlike CFC systems where each PMT voltage can be controlled in order to maximise signal resolution, we have no such controls over individual camera channels. As such, we rely on altering laser powers to control signal. PI is maximally excited by a 488 nm laser line but is also excited by a 405 nm laser to ~20 % of maximum. If the PI intensity is very bright due to over-staining then the 488 nm laser output would need to be set comparatively low. If the CTV or other 405 nm excited fluorochromes require the 405 nm laser to be set near to maximum then there is a possibility that the 405 nm excitation of PI will exceed the 488 nm excitation meaning that PI is now maximally fluorescent in CH11. This is because the spectral widths of CH5 and CH11 are identical to one another (*see* Fig. 1). CH11 would not be usable now to detect AF-647 or equivalent dyes.
10. The sample run rate of any IFC system is slower compared to a CFC system. The overriding consideration for sample speed is image quality and the system ensures that the objects move past the camera at the appropriate rate. The CCD camera has no electronic dead time, therefore concentrating the sample into a density such as  $20\text{--}30 \times 10^6$  cells/ml means that the sample run time is greatly reduced but there will be no detrimental effect on the data.
11. As IDEAS has no modelling options for either cell cycle or proliferation, one can export the data as an FCS file and use FlowJo for analysis. In this instance, IDEAS can be used to compensate data and to perform limited data reduction (single in focus cells for example) as well as any custom masks and feature extraction. We have found that FlowJo X handles IFC-derived FCS files particularly well due to the easy re-scaling feature within the software. However if the values of the exported features map between 0 and 1, or go into the negatives, there can be issues with displaying the data. FCS Express (DE Novo, USA) can also be used and does not suffer from this scaling issue.
12. CFC has had significant impact on the area of cell cycle analysis [24]. It is essential to consider that within an asynchronously growing population, not only will cells be in different stages of a given cell cycle; they may also temporally separated across multiple division rounds. As a result, the analysis of asynchronously growing cells always requires a multiparameter, temporal approach. One particularly powerful solution to this issue is

the bromodeoxyuridine (BrdU)-Hoechst quenching method whereby defined populations can be tracked from their origin at the point of drug addition as long as BrdU is added at the exact same moment [25]. However the technique limited by incompatibility with antigen staining, resolution of up to 2 two division rounds and a very complex analysis framework. In our hands, the use of CTV, PI, and MPM2 is a viable alternative. While our approach may lack the ability to track specific cohorts from defined cell cycle stages through progressive rounds, it does provide the temporal data required to elucidate the true impact a drug may exert intra- and inter-cycle while still being relatively simple to perform. By adopting an analysis strategy that is in effect a hybrid between the classical dye dilution calculation of precursory frequencies [26] and traditional cell cycle phase modeling, we can express the data as the frequency of the input population within a given cell cycle phase within each division round.

13. We have found that the overall masking modification string is similar (“threshold” followed by “range”) whether images are collected at 20×, 40×, or 60× magnification. However the absolute threshold values for the adaptations will differ. For example images collected at 60× will have more pixel texture and information associated with them. As such, thresholding tends to form smaller satellite masks, especially with highly textured prophase nuclei. This requires a more stringent range mask adaptation based on the minimum masking area. The performance and appropriateness of a particular mask is also related to the staining quality in a given channel. We find that masks not only need to be adapted between magnifications but also between experiments due to differences in the signal to noise within the image. Therefore we suggest that the exact composition of the masking adaptations should be determined empirically.
14. One of the biggest challenges to analysing cells in telophase is misclassifying due to the presence of doublet cells. The fact that MPM2 is still able to identify telophasic cells above background levels of fluorescence is a massive improvement on our previous approach that relied on complex masking without a unique fluorescence signal [16]. There is still a possibility that two conjoined metaphase cells (MPM2 positive and with flat nuclei) could resemble a telophasic cell but as MPM2 is brighter on metaphase cells, one could use this feature to exclude such a possibility. Moreover an actual telophase cell would still have the same sum CTV intensity as a single undivided cell, simply distributed over two poles. The only remaining issue is if two cells from round 1 became attached they could look like a telophase cells about to divide for the first

time but if the population is analysed before cells enter the second mitosis, then these cells will not be present.

15. It has recently been suggested that all cellular protein is naturally inherited in an asymmetrical fashion within T cells and that this needs to be factored in to any dye-dilution-based calculations of cell division [27, 28]. We have specifically looked at CFSE and CTV inheritance across the plane of cytokinesis in telophasic cells and found no evidence of any asymmetrical inheritance [19] outside of what can be considered normal stochastic variation in the inheritance of protein [29]. We use this to define the “normal” limits of protein inheritance and to therefore define the boundary of asymmetry. We have termed this a “morphometrically relevant biological control” (MRB) [21] as like a fluorescence minus one control (FMO) in CFC is used to determine negative and positive thresholds based on gross fluorescence, it can be used to set gating thresholds on a spatial/morphometric parameter. The idea of an MRB control has been used successfully in other IFC-based analysis [30]. There has been some recent question over the validity of using a diffuse CTV signal to control for a more discrete signal [31]. However we have also stain for endosomes (discrete signal) and shown they are inherited in symmetrical fashion same as diffuse signal [16].
16. We have found that for telophasic cells, if one pole is imaged outside of the focal plane then this can affect the measurement of signal, tending to then lead to misclassifying a cell as asymmetric. However, the same effect is seen when one looks at the PI and CTV signals placing them outside the limits of symmetry. We therefore reject these cells from the analysis based on the MRB controls (PI/CTV). It has been suggested that the extended depth of field option (EDF) could be employed to overcome issues with defocus by bringing all light back into the focal plane [32]. However as we have no control over the orientation of a telophasic cell as it flows, EDF would simply merge the fluorescent information from two poles imaged at an angle as they are brought in to the same focal plane by deconvolution. Therefore the defocus information gained by acquiring in non-EDF mode can be used to ones advantage in this regard to eliminate cells with one or more poles outside the focal plane.

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