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An Automated Method To Perform The In Vitro Micronucleus Assay using Multispectral Imaging Flow Cytometry

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Dr. Nandita Sing
Senior Science Editor
Journal of Visualized Experiments

October 30, 2018

Dear Dr. Singh

I am pleased to submit an Invited Methods Article entitled “Automation of the *In Vitro* Micronucleus Assay using Multispectral Imaging Flow Cytometry” for consideration for publication in the *Journal of Visualized Experiments (JoVE*). This manuscript describes, in detail, the methodology employed to perform an automated multispectral imaging flow cytometry (MIFC) based micronucleus (MN) assay in toxicology that I published in *Cytometry Part A* in 2018. Additionally, my collaborators and I have published several other papers in a number of peer reviewed journals describing the development of this method for radiation biodosimetry.

In this manuscript, I outline all required steps to perform the laboratory protocol as well as the data acquisition and analysis for the *in vitro* MN assay in toxicology using MIFC. All data analysis has been automated using the software that accompanies the MIFC and a full, step-by-step protocol is presented here, such that other researchers can repeat and verify the effectiveness of the protocol and validate the results. The brief summary of results in the manuscript indicate that this method is able to detect statistically significant increases in MN formation following exposure of TK6 cells to well-known aneugenic and clastogenic agents.

I believe that this manuscript is well suited for publication in *JoVE* since the laboratory protocol is elegantly simple but the data analysis is very complicated. As such, having a step-by-step document that researchers can follow to create data analysis templates in their own laboratories will be very helpful. Furthermore, the manuscript contains explanations on the development of this new methodology to perform automated analysis of MN data and these details will aid readers who are interested in adopting this technique in their laboratories.

This manuscript has not been published and is not under consideration for publication elsewhere. Where figures have been modified from previous publications, the appropriate permissions have been obtained. I must disclose that I am employed by MilliporeSigma, the maker of the Amnis® brand ImageStream® MIFC used in this work. This disclosure is declared in the manuscript.

Thank you for your consideration!

Sincerely,

Matthew A. Rodrigues, PhD
Research Scientist
MilliporeSigma, Seattle, WA, USA

TITLE:

An Automated Method to Perform The In vitro Micronucleus Assay using Multispectral Imaging Flow Cytometry

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KEYWORDS:
TK6 cells, genotoxicity, cytotoxicity, in vitro micronucleus assay, imaging flow cytometry, image analysis, automation, multispectral imaging flow cytometry

SUMMARY:
The in vitro micronucleus assay is a well-established method for evaluating genotoxicity and cytotoxicity but scoring the assay using manual microscopy is laborious and suffers from subjectivity and inter-scorer variability. This paper describes the protocol developed to perform a fully automated version of the assay using multispectral imaging flow cytometry.

ABSTRACT:
The in vitro micronucleus (MN) assay is often used to evaluate cytotoxicity and genotoxicity but scoring the assay via manual microscopy is laborious and introduces uncertainty in results due to variability between scorers. To remedy this, automated slide-scanning microscopy as well as conventional flow cytometry methods have been introduced in an attempt to remove scorer bias and improve throughput. However, these methods have their own inherent limitations such as inability to visualize the cytoplasm of the cell and the lack of visual MN verification or image data storage with flow cytometry. Multispectral Imaging Flow Cytometry (MIFC) has the potential to overcome these limitations. MIFC combines the high resolution fluorescent imagery of microscopy with the statistical robustness and speed of conventional flow cytometry. In addition, all collected imagery can be stored in dose-specific files. This paper describes the protocol developed to perform a fully automated version of the MN assay on MIFC. Human lymphoblastoid TK6 cells were enlarged using a hypotonic solution (75 mM KCl), fixed with 4% formalin and the nuclear content was stained with Hoechst 33342. All samples were run in suspension on the MIFC, permitting acquisition of high resolution images of all key events required for the assay (e.g. binucleated cells with and without MN as well as mononucleated and polynucleated cells). Images were automatically identified, categorized and enumerated in the MIFC data analysis software, allowing for automated scoring of both cytotoxicity and genotoxicity. Results demonstrate that using MIFC to perform the in vitro MN assay allows statistically significant increases in MN frequency to be detected at several different levels of cytotoxicity when compared to solvent controls following exposure of TK6 cells to mitomycin C and colchicine, and that no significant increases in MN frequency are observed following

exposure to mannitol.

INTRODUCTION

The in vitro micronucleus (MN) assay is a commonly used test to assess cytotoxicity and genotoxicity as a screening tool in several fields of study such as chemical and pharmaceutical development as well as human biomonitoring among individuals exposed to various environmental, occupational or lifestyle factors[1-3]. MNs consist of chromosome fragments or whole chromosomes generated during cell division that are not incorporated into one of the two main daughter nuclei. Following telophase, this chromosomal material forms into an individual, rounded body inside the cytoplasm that is separate from either of the main nuclei[2]. Therefore, MNs are representative of DNA damage and have been used for many years as an endpoint in genotoxicity testing[4]. The most appropriate method to measure MN is the cytokinesis-block micronucleus (CBMN) assay. Using the CBMN assay, the frequency of MN in binucleated cells (BNCs) can be scored by incorporating cytochalasin B (Cyt-B) into the sample. Cyt-B allows nuclear division but prevents cellular division and thus, restricts scoring of MN to BNCs that have divided only once[5].

Protocols using both microscopy and flow cytometry have been developed and validated and are routinely used to perform the in vitro MN assay[6-14]. Microscopy benefits from being able to visually confirm that MNs are legitimate but is time consuming and prone to variability between scorers[15]. To address this, automated microscopy methods were developed to scan slides and capture images of nuclei and MN[16-19], but the cytoplasm cannot be visualized, making it difficult to determine if an MN is actually associated with a specific cell. Furthermore, these methods have difficulties identifying polynucleated (POLY) cells (including tri- and quadranucleated cells) which are required for the calculation of cytotoxicity when using Cyt-B[9]. Flow cytometry methods developed to perform the MN assay employ fluorescence as well as forward and side scatter intensities to identify populations of both the nuclei and MN that have been liberated from the cell following lysis[20-22]. This allows data to be acquired from several thousand cells in a few minutes and permits automated analysis[23]; however, the inability to visualize the cells makes it impossible to confirm that scored events are genuine. Additionally, lysing the cell membrane inhibits the use of Cyt-B as well as creating a suspension that contains other debris such as chromosome aggregates or apoptotic bodies and there is no way to differentiate these from MN[24].

In light of these limitations, Multispectral Imaging Flow Cytometry (MIFC) is an ideal system to perform the MN assay since it combines the high resolution fluorescent imagery of microscopy with the statistical robustness and speed of conventional flow cytometry. In MIFC, all cells are introduced into a fluidics system and are then hydrodynamically focused into the center of a flow cell cuvette. Orthogonal illumination of all cells is accomplished through the use of a brightfield (BF) light-emitting diode (LED), a side scatter laser and (at least) one fluorescent laser. Fluorescent photons are captured by one of three (20x, 40x or 60x) high numerical aperture objective lenses and then pass through a spectral decomposition element. Photons are then focused onto a charge-coupled device (CCD) camera to obtain high resolution images of all cells that pass through the flow cell. To avoid blurring or streaking, the CCD operates in time delay

integration (TDI) mode which tracks objects by transferring pixel content from row to row down the CCD in synchrony with the velocity of the cell in flow. Pixel information is then collected from the last row of pixels. TDI imaging combined with spectral decomposition allows up to 12 images (2 BF, 10 fluorescent) to be captured simultaneously from all cells passing through the flow cell. All captured imagery is stored in sample-specific data files, permitting analysis to be performed at any time using the MIFC data analysis software. Finally, data files retain the link between cellular images and dots on all bivariate plots. This means that any dot on a traditional bivariate plot can be highlighted and its corresponding BF and fluorescent imagery will be displayed[25].

Recently, MIFC-based methods have been developed to perform the MN assay for both triage radiation biodosimetry[26-30] and genetic toxicology[31,32] testing. This work has demonstrated that cellular images of main nuclei, MN and the cytoplasm can be imaged with higher throughput than other methods[26]. All cell types required for analysis, including MONO cells, BNCs (with and without MN), and POLY cells, can be automatically identified in the MIFC data analysis software, and implementation of the scoring criteria developed by Fenech et al. is accomplished through the use of various mathematical algorithms[6,33]. Results from biodosimetry showed that dose response calibration curves were similar in magnitude to those obtained from other automated methods in the literature when quantifying the rate of MN per BNC[29]. Additionally, recent work in toxicology demonstrated that images of MONO cells, BNCs (with and without MN) and POLY cells can be automatically captured, identified, classified and enumerated using MIFC. The protocol and data analysis enabled the calculation of cytotoxicity and genotoxicity after exposing TK6 cells to several clastogens and aneugens[31].

The protocol presented in this paper describes a method to perform the in vitro MN assay using MIFC. The sample processing technique used in this work requires less than 2 h to process a single sample and is relatively easy to perform in comparison to other methods. The data analysis in the MIFC analysis software is complicated, but creation of the analysis template can be accomplished in a few hours following the steps outlined in this paper. Moreover, once the template has been created, it can be automatically applied to all collected data without any further work. The protocol outlines all steps required to expose TK6 cells to clastogens and aneugens, describes how to culture, process and stain the cells, and demonstrates how to acquire high resolution imagery using MIFC. Furthermore, this paper illustrates the current best practices for analyzing data in MIFC software to automatically identify and score MONO cells, BNCs, and POLY cells for the purposes of calculating both cytotoxicity and genotoxicity.

PROTOCOL

1. Preparation of culture medium and culturing of TK6 cells

NOTE: Some chemicals used in this protocol are toxic. Inhaling, swallowing or contacting skin with cytochalasin B can be fatal. Wear appropriate personal protective equipment including a laboratory coat and two pairs of nitrile gloves. Wash hands thoroughly after handling. Formalin/formaldehyde is toxic if inhaled or swallowed; is irritating to the eyes, respiratory system, and skin; and may cause sensitization by inhalation or skin contact. There is a risk of

serious damage to eyes. It is a potential carcinogen.

1.1. Prepare 565 mL of 1x RPMI culture medium. Add 5 mL of MEM non-essential amino acids (100x), 5 mL of sodium pyruvate (100 mM), 5 mL of penicillin-streptomycin-glutamine (100x), and 50 mL of fetal bovine serum (FBS) to a 500 mL bottle of 1x RPMI 1640 medium. Prepare the medium in a biosafety cabinet and store at 2–8 °C. Heat the medium to 37 °C prior to adding it to the TK6 cells (see **Table of Materials**).

1.2. Thaw 1 mL of TK6 cells (stored at -80 °C in DMSO) in 10 mL of medium. Centrifuge the cells at 200 x *g* for 8 min and aspirate the supernatant. Transfer the cells to 50 mL of media and incubate at 37 °C, 5% CO_2. The doubling time of TK6 cells varies from ~12–18 h and a few (3 or 4) passages will be required for the cells to reach their maximum proliferation rate (doubling time of about 14–15 h; see **Table of Materials**).

1.3. Culture 100 mL of cells to a concentration of ~7–8 x 10^5 cells/mL.

2. Preparation of clastogens and/or aneugens and cytochalasin B

2.1. Prepare appropriate stock concentrations of desired clastogens and aneugens. For example, for mitomycin C, dissolve a full 2 mg bottle in 10 mL of sterile water to achieve a final stock concentration of 200 µg/mL. Mitomycin C can be stored at 4 °C for three months (see **Table of Materials**).

2.2. On experiment day, prepare dilutions of the desired chemicals that are either 10-fold or 100-fold higher than the desired exposure concentrations if diluting in sterile water or DMSO, respectively.

2.3. For mitomycin C, prepare 3 mL dilutions in sterile water of 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 µg/mL. For colchicine, prepare 3 mL dilutions in sterile water of 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 µg/mL. Finally, for mannitol, prepare 3 mL dilutions in sterile water of 5, 10, 20, 30, 40, and 50 mg/mL.

2.4. Prepare a 200 µg/mL stock concentration of cytochalasin B by dissolving a 5 mg bottle into 25 mL of DMSO. cytochalasin B can be stored at -20 °C for several months.

3. Exposure of cells to clastogens and/or aneugens

3.1. Add 1 mL of desired chemical (e.g. mitomycin C) to 9 mL of cells at ~7-8x10^5 cells/mL in a T25 flask. For the control samples, add 1 mL of sterile water. Place the flasks in a 37 °C, 5% CO_2 incubator for 3 h.

NOTE: If chemicals are diluted in DMSO, add only 100 µL of the chemical to each flask and add 100 µL of DMSO to controls. Each flask should contain 9.900 mL of cells.

3.2. After 3 h, remove flasks from the incubator and transfer cells to 15 mL polypropylene tubes. Centrifuge at 200 x *g* for 8 min, aspirate the supernatant and transfer cells to new T25 flasks containing a total of 10 mL of fresh culture medium. Add 150 µL of the stock concentration (200 µg/mL) of cytochalasin B to each flask to achieve a final concentration of 3 µg/mL.

3.3. Return the flasks to the 37 °C, 5% CO_2 incubator for a recovery time equal to 1.5–2.0 doubling times, as recommended by the OECD guidelines[9]. For the TK6 cells used in this work, the recovery time was 24 h.

NOTE: The doubling time of the TK6 cells used here was 15 h and a recovery time of 24 h (1.6 doubling times) was used. Recovery times less than 1.5 doubling times will reduce proliferation in samples exposed to higher doses impacting the number of BNCs. Conversely, recovery times of more than 2.0 will produce a disproportionate number of polynucleated cells in control samples, skewing cytotoxicity calculations.

4. Preparation of buffers for fixation and labeling of DNA content (see **Table of Materials**)

4.1. Prepare 75 mM of potassium chloride (KCl) by adding 2.79 g to 500 mL of ultrapure water. Stir the solution for 5 min using a magnetic stirrer and sterile filter through a 200 µm filter. The 75 mM KCl solution can be stored at 4 °C for several months.

4.2. Prepare a sufficient amount of 4% formalin for the experiment, anticipating that a total of 2.1 mL must be added to each sample. For example, to prepare 10 mL of 4% formalin, add 4 mL of 10% formalin stock to 6 mL of 1x Dulbecco's Phosphate-Buffered Saline solution without Ca^{2+} or Mg^{2+} (PBS). This 4% formalin can be stored at room temperature for several weeks.

4.3. Prepare 510 mL of wash buffer (2% FBS in 1X PBS) by adding 10 mL of FBS to a 500 mL bottle of 1x PBS.

4.4. Prepare 10 mL of a 100 µg/mL concentration of Hoechst 33342 by adding 100 µL of the stock concentration (1 mg/mL) to 9,900 µL of 1X PBS. The Hoechst 33342 solution can be stored at 4 °C for several months.

5. Sample processing: hypotonic swelling, fixation, cell counting and labeling DNA content

5.1. At the end of the recovery period, remove all flasks from the incubator and transfer all samples to 15 mL polypropylene tubes. Centrifuge all samples at 200 x *g* for 8 min.

5.2. Aspirate the supernatant, resuspend the cells and add 5 mL of 75 mM KCl. Mix gently by inversion three times and incubate at 4 °C for 7 min.

5.3. Add 2 mL of 4% formalin to each sample, mix gently by inversion three times and incubate at 4 °C for 10 min. This step acts as a “soft fixation”.

5.4. Centrifuge all samples at 200 x *g* for 8 min. Aspirate the supernatant and resuspend in 100 µL of 4% formalin for 20 min. This step acts as a "hard fixation".

5.5. Add 5 mL of wash buffer and centrifuge at 200 x *g* for 8 min. Aspirate the supernatant and resuspend in 100 µL of wash buffer.

5.6. Transfer all samples to 1.5 mL microcentrifuge tubes.

5.7. Perform a cell count on each sample to determine the number of cells per sample. Samples will be highly concentrated so a 1:100 dilution in 1x PBS (10 µL of sample in 990 µL of PBS) will likely be required to obtain an accurate count.

NOTE: At this point it is best to perform cell counts using a hemocytometer. Adding KCl gives the cytoplasm a translucent appearance, making it difficult for automated cell counters to recognize them. Also, automated counters have difficulty scoring polynucleated cells due to their size.

5.8. If not running the samples on the MIFC immediately, they can be stored at 4 °C for several days. When ready to run samples, add 5 µL of 100 µg/mL per $1x10^6$ cells/mL to each sample. Also add 10 µL of 500 µg/mL of RNase per 100 µL of sample for a final concentration of 50 µg/mL. Incubate the samples at 37 °C, 5% CO_2 for 30 min.

5.9. Micro-centrifuge all samples at 200 x *g* for 8 min and use a pipette to remove the supernatant leaving ~30 µL. Use a pipette to resuspend all samples before running on the MIFC ensuring there are no bubbles in the tube. **Do not vortex.**

6. Starting and calibrating the MIFC

6.1. Ensure the sheath, system calibration reagent, debubbler, cleanser and sterilizer containers are full and the waste tank is empty. Power up the system and double-click on the MIFC software icon. Click the Startup button and make sure that the **Start all calibrations and tests** checkbox is checked. This will flush the system, load sheath and system calibration reagents, and calibrate the system (see **Table of Materials**).

7. Running samples on the MIFC

NOTE: This section assumes the use of a 2 camera MIFC. If using a 1 camera MIFC, please see Supplement 1 – Full Protocol, section 7 for the creation of plots during acquisition

7.1. Launch the MIFC data acquisition software (see **Table of Materials). Figure 1** shows the instrument settings. Turn on the 405 nm laser and set the laser power to 10 mW (**A**). Disable all other lasers (including SSC) and set the BF to channels 1 and 9 (**B**). Confirm that the magnification slider is set to 60x (**C**), high-sensitivity mode is selected (**D**), and that only channels 1, 7, and 9 are showing in the image gallery.

7.2. Click on the **Scatterplot** icon. Select the **All** population and select **Area M01** on the X-axis and **Aspect Ratio M01** on the Y-axis. Click on the **Square Region** icon and draw a region around the single cells. Name this region **Single Cells**. Right click on the plot and select **Regions**. Highlight the **Single Cells** region and change the x-coordinates to 100 and 900 and change the y-coordinates to 0.75 and 1 (**Figure 1I**).

7.3. Click on the **Scatterplot** icon. Select **Single Cells** as the parent population, select **Gradient RMS M01** on the X-axis, and **Gradient RMS M07** on the Y-axis. Click on the **Square Region** icon and draw a region around the majority of the cells. Name this region **Focused Cells**. Right click on the plot and select **Regions**. Highlight the **Focused Cells** region and change the x-coordinates to 55 and 75 and change the y-coordinates to 9.5 and 20 (**Figure 1J**).

7.4. Click on the **Histogram** icon. Select the **Focused Cells** population and select **Intensity M07** as the feature. Click on the **Linear Region** icon and draw a region across the main peak in the histogram. Name this region **DNA-positive**. Right click on the plot and select **Regions**. Highlight the **DNA-positive** region and change the coordinates to 2×10^5 and 2×10^6. The range may have to be adjusted depending on intensity peak on the histogram (**Figure 1K**).

7.5. Set the acquisition parameters (**Figure 1E**). Specify the file name and the destination folder, change the number of events to 20,000, and select the **DNA-positive** population.

7.6. Click **Load** (**Figure 1F**) and place the control sample into the MIFC. Click the **Acquire** button to collect the data (**Figure 1G**). Once the acquisition is complete, click the **Return** button to return the sample (**Figure 1H**). Remove the sample tube from the instrument. Repeat this process for all remaining samples in the experiment.

8. Opening a data file in IDEAS

8.1. Launch the MIFC analysis software package (see the **Table of Materials**). Click on Start Analysis to start the **Open File Wizard**. Select a data file by browsing to the desired raw image file (.rif). Click the **Open** button and click **Next**.

8.2. Since this is a single color assay, compensation is not necessary so click **Next** to bypass the compensation step. At this stage there is no analysis template to apply, so click **Next** again. If the analysis template downloaded from the supplementary material, select it now. These templates only work with a 2 camera MIFC with BF set to channels 1 and 9 and nuclear imagery in channel 7 during acquisition.

8.3. By default, the .cif and .daf file names are automatically generated to match the .rif. **It is not recommended to change the names of the .cif and .daf**. Click **Next**. Set the image display properties by selecting the **01** and **07**. Click **Next**. There is no wizard for this application, so click **Finish**. It is very important to save the data analysis file (.daf) and the analysis template (.ast) often during sections 9–14 to avoid loss of progress.

9. Creating masks and features to identify BNCs

9.1. Click on the **Image Gallery Properties** icon (blue/white icon). In the **Display Properties** tab click **Set Range to Pixel Data** then change the color to yellow. Click **OK**. Hoechst images are now easier to view against the black background.

9.2. Create the non-apoptotic cells plot.

9.2.1. Click on the **Analysis** tab, then click **Masks**. Click **New** then click **Function**. Under **Function** choose **Threshold**, under **Mask** choose **M07** and set the **Intensity Percentage** to 50. Click **OK** then **OK** again. Click **Close**.

9.2.2. Click on the **Analysis** tab, click **Features**, then click **New**. For the **Feature Type** select **Area**. For **Mask** select the **Threshold(M07,Ch07,50)**. Click **Set Default Name** and click **OK**. Click **Close** to start calculating the feature values.

9.2.3. Click on the **Dot Plot** icon. Select the **All** population. For the **X-axis feature** choose the **Contrast_M01_Ch01** feature and for the **Y-axis feature** choose **Area_Threshold(M07,Ch07,50)**. Click **OK**.

9.2.4. Click the **Square Region** button and draw a region around the majority of the cells. Call this region **Non-apoptotic**. Right click on the plot and click **Regions**. Highlight the **Non-apoptotic** region. Set the **x-coordinates** to 0 and 15 and set the **y-coordinates** to 50 and 300. Click **Close**.

9.3. Create the BNC mask (steps 9.3.1–9.3.5) to identify cells that contain only two nuclei.

9.3.1. Browse for a BNC in the image gallery and click on it. This is to visualize creation of the mask in the Hoechst channel.

9.3.2. Click on the **Analysis** tab, then click **Masks**. Click **New** then click **Function**. Under **Function** choose **LevelSet**, under **Mask** choose **M07**, select the **Middle Level Mask** radio button, and set the **Contour Detail Scale** to 3.00. Click **OK** then **OK** again.

9.3.3. Click **New** then click **Function**. Under **Function**, choose **Dilate**, and under **Mask** choose **LevelSet(M07,Ch07,Middle,3)**. Set the **image to display** to **Ch07**, and set the **Number of Pixels** to 2. Click **OK** then **OK** again.

9.3.4. Click **New** then click **Function**. Under **Function** choose **Watershed**, and under **Mask** choose **Dilate(LevelSet(M07,Ch07,Middle,3)2)**. Set the **image to display** to **Ch07**, and set the **Line Thickness** to 1. Click **OK** then **OK** again.

9.3.5. Click **New** then click **Function**. Under **Function** choose **Range**, under **Mask** choose **Watershed(Dilate(LevelSet(M07,Ch07,Middle,3)2))**. Set the **image to display** to **Ch07**. Set the minimum and maximum area values to 115 and 5000, respectively. Set the minimum and

maximum aspect ratio values to 0.4 and 1, respectively. Click **OK.** In the **Name** field change the text to read **BNC** then click **OK**.

9.4. Create the features and plots to obtain the final BNC population

9.4.1. Spot Count BNC feature: Click on the **Analysis** tab, then **Features**, then **New**. For the **Feature Type** select **Spot Count**. For **Mask**, select the final BNC mask created in 9.3.5. Set the **Connectedness** to **Four** and change the name to **Spot Count BNC**. Click **OK** then **Close** to calculate the feature values.

9.4.2. Spot Count BNC histogram. Click the **Histogram** icon. Select **Non-apoptotic** as the parent population. For the **X-axis feature** choose the **Spot Count BNC** feature. Click **OK**. Click on the Linear Region icon. Draw a region across bin **2**. Call this region **2N**.

NOTE: Refer to section 9 in Supplement 1 – Full Protocol to create the remaining masks, features and plots to identify the final BNC population

10. Creating masks and features to identify MN within the BNC population

10.1. Create the MN mask. Browse for a BNC that contains an MN in the image gallery and click on it. This is to visualize creation of the MN mask in the Hoechst channel. Click on the **Analysis** tab, then click **Masks**.

10.1.1. Create Spot Identification Mask 1:

10.1.1.1. Click **New** then click **Function**. Under **Function** choose **Spot** and ensure the **Bright** radio button is selected. Under **Mask** choose **M07**, set the **Spot to Cell Background Ratio** to **2.00**. Set the **Minimum Radius** to **2** and the **Maximum Radius** to **6**. Click **OK** then **OK** again.

10.1.1.2. Click **New** then click **Function**. Under **Function** choose **Range**, and under **Mask** choose **LevelSet(M07,Ch07,Middle,3)**. Set the **Image to display** to **Ch07**. Set the **Minimum and Maximum Area** to 80 and 5000, respectively. Set the **Minimum** and **Maximum Aspect Ratio** to 0 and 1, respectively. Click **OK** then **OK** again.

10.1.1.3. Click **New** then click **Function**. Under **Function** choose **Dilate**, under **Mask** choose **Range(LevelSet(M07,Ch07,Middle,3),80-5000,0-1)**. Set the **Image to display** to **Ch07**. Set the **Number of Pixels** to **2**. Click **OK** then **OK** again.

10.1.1.4. Click **New**. Double Click on the **Spot(M07, Ch07, Bright, 2, 6, 2)** mask to add it to the mask definition. Click the **And** operator and then the **Not** operator. Double click the **Dilate(Range(LevelSet(M07, Ch07, Middle, 3), 80-5000, 0-1), 2)** mask to add it to the mask definition. Click **OK**.

10.1.1.5. Click **New**, then **Function**. Under **Function** choose **Range** and under mask choose the

mask created in 10.1.1.4:

10.1.1.5.1. Select **Spot(M07, Ch07, Bright, 2, 6, 2)** And Not **Dilate(Range(LevelSet(M07, Ch07, Middle, 3), 80-5000, 0-1), 2)**.

10.1.1.5.2. Set the **Image to Display** to **Ch07**. Set the **Minimum and Maximum Area** to 10 and 80, respectively. Set the **Minimum and Maximum Aspect Ratio** to 0.4 and 1, respectively. Click **OK** then **OK** again. Spot identification mask 1 is complete.

NOTE: Refer to section 10 in Supplement 1 – Full Protocol to create the masks, features and plots to identify the final MN population

11. Create masks, features and plots to identify the Mononucleated and Polynucleated populations

11.1. Create the POLY mask. Click **Analysis**, then **Masks**, then **New** then **Function**. Under **Function** choose **Range**, under **Mask** choose **Watershed(Dilate(LevelSet(M07, Ch07, Middle, 3), 2))**. Set the **image to display** to **Ch07**. Set the **Minimum** and **Maximum Area** values to 135 and 5000, respectively. Set the **Minimum** and **Maximum Aspect Ratio** values to 0.4 and 1, respectively. Click **OK**. In the **Name** field, change the text to read **POLY** then click **OK** then **Close**. The Polynucleated cell mask is complete.

11.2. Create the POLY Component Masks.

11.2.1. POLY Component Mask 1: Click on the **Analysis** tab, then **Masks**, then **New**, then Function. Under **Function** select **Component**, and under **Mask** select the **POLY** mask. For **Ranking Feature** select **Area**, and for **Sorting Order** click the **Descending** radio button. Set **Rank** to **1**. Click **OK** then **OK** again.

11.2.2. POLY Component Masks 2, 3 and 4: Repeat all steps in 11.2.1 except set **Rank** to **2**, **3** and **4** to create the individual component masks.

11.3. Spot Count using POLY mask.

11.3.1. Click the **Analysis** tab, then **Features**, then **New**. For **Feature Type**, select **Spot Count**. For **Mask** choose the **POLY** mask and set the Connectedness at **4**. Click **Set Default Name** and click **OK** then **Close** to calculate the feature values.

11.3.2. Click the Histogram icon. Select the **Non-apoptotic** population. For the **X-axis feature** choose the **Spot Count_POLY_4** feature.

11.3.3. MONO spot count region. Click on the Linear Region icon. Draw a region across bin **1** on the histogram created in 11.3.2. Call this region **1N**.

11.3.4. TRI spot count region. Click on the Linear Region icon. Draw a region across bin **3** on the histogram created in 11.3.2. Call this region **3N**.

11.3.5. QUAD MONO spot count region. Click on the Linear Region icon. Draw a region across bin **4** on the histogram created in 11.3.2. Call this region **4N**.

11.4. Identify the MONO population.

11.4.1. Create the MONO Aspect Ratio Feature. Click the **Analysis** tab, then **Features**, then **New**. Under **Feature Type**, select the **Aspect Ratio** feature and under **Mask** select **Component(1, Area, POLY, Descending)**. Click **Set Default Name** then click **OK**.

11.4.2. Create the MONO Circularity Feature. With the Feature Manager window still open, click **New**. Under **Feature Type**, select the **Circularity** feature and under **Mask** select **Component(1, Area, POLY, Descending)**. Click **Set Default Name** then click **OK** then click **Close** to calculate the feature values.

11.4.3. For the circular MONO cells dot plot, click the **Dot Plot** icon. Select **1N** as the parent population. For the **X Axis Feature** choose **Circularity_Component(1, Area, POLY, Descending)** and for the **Y Axis Feature** choose **Aspect Ratio_Component(1, Area, POLY, Descending)**. Click **OK**. Click the **Square Region** button and draw a region around the cell population towards the top right portion of the plot. Name this region **Circular_1N**. Right click on the plot and click **Regions**. Highlight the **Circular_1N** region. Change the **X Coordinates** to 20 and 55 and change the **Y Coordinates** to 0.85 and 1.0. Click **Close**.

11.4.4. Create the Area POLY/Area_M07 feature. Click the **Analysis** tab, then **Features**, then **New**. Under **Feature Type**, select the **Area** feature and under **Mask** select **Component(1, Area, POLY, Descending)**. Click **Set Default Name** then click **OK**.

11.4.5. With the Feature Manager window still open, click **New** then under Feature Type click the **Combined** radio button. From the list of features, highlight the **Area_Component(1, Area, POLY, Descending)** and click the down arrow to add it to the feature definition. Click the division symbol (/). Select the Area_M07 feature and click the down arrow to add it to the feature definition. Click **Set Default Name** and click **OK**. Click **Close** to start calculating the feature values.

11.4.6. For the final MONO population dot plot, click the **dot plot** icon. Select **Circular_1N** as the parent population. For the **X Axis Feature** choose **Aspect Ratio_M07** and for the **Y Axis Feature** choose **Area_Component(1, Area, POLY, Descending) / Area_M07**. Click **OK**. Click the **Square Region** button and draw a region around the majority of the cells. Name this region **Mononucleated**. Right click on the plot and click **Regions**. Highlight the **Mononucleated** region. Change the **X Coordinates** to 0.85 and 1.0 and change the **Y Coordinates** to 0.55 and 1.0. Click **Close**.

NOTE: Refer to section 11 in Supplement 1 – Full Protocol to create the masks, features and plots

to identify the final trinucleated and polynucleated populations.

12. Create a custom view to examine the BNC and MN masks

12.1. Click on the **Image Gallery Properties** button then click on the **View** tab. Click on the **Composites** tab then click **New**. Under **Name** type **Ch01/Ch07**. Click **Add** Image. Under **Image** choose **Ch01** and set the **Percent** to 100. Click **Add Image** again, under **Image** choose **Ch07** and set the **Percent** to 100.

12.2. Click **New** and under Name type **BNC and MN masks**

12.3. Click **Add Column**. Under **Image Type** choose **Ch01** and under **Mask** choose **None**

12.4. Click **Add Column**. Under **Image Type** choose **Ch07** and under **Mask** choose **None**

12.5. Click **Add Column**. Under **Image Type** choose **Ch07** and under **Mask** choose **BNC**

12.6. Click **Add Column**. Under **Image Type** choose **Ch07** and under **Mask** choose **MN mask**

12.7. Click **Add Column**. Under **Image Type** click the **Composite** radio button. The **Ch01/Ch07** composite image should be automatically added to the view. Click **OK** to close the Image Gallery Properties window.

13. Create a custom view to examine the POLY mask

13.1. Refer to section 13 in Supplement 1 – Full Protocol to create a custom view to examine the POLY mask

14. Create a statistics table to enumerate key events

14.1. Click on the **Reports** tab then click **Define Statistics Report**. In the new window, click Add **Columns**.

14.2. Add the BNC count statistic. Under **Statistics** select **Count** and under **Selected Population** choose the **BNCs** population. Click **Add Statistics** to add the statistic to the list.

14.3. Repeat step 14.2 to create separate columns for the **MN BNCs**, **MONO**, **TRI** and **POLY** populations. Click **Close** then click **OK**.

14.4. The data analysis template is complete (**Figure 2**). Save the template (File, Save as Template). The full mask list can be found in **Supplement 2 – Mask List**.

15. Batch process experiment files using the data analysis template

15.1. Under the **Tools** menu click **Batch Data Files** then click **Add Batch** in the new window.

15.2. In the new window click **Add Files** to select the experiment files (.rif) to add to the batch. Under the **Select a template or data analysis file (.ast, .daf)** option, click the open folder icon to browse to and open the data analysis template (.ast file) that was saved in step 14.4.

15.3. Click the **Preview Statistics Report** button to preview the statistics table. No values will be displayed here since they have not yet been calculated. However, this step serves as a check to ensure the proper analysis template has been selected prior to running the batch.

15.4. Click **OK** to close the current window. Then click **Submit Batches** to start the batch processing of all files.

15.5. When the batch processing has completed, a .txt file will be available in the folder that contains all of the .rif files. Use these statistics to calculate genotoxicity and cytotoxicity.

16. Calculating the genotoxicity and cytotoxicity parameters

16.1. Calculating genotoxicity: To calculate genotoxicity, use the statistics table created in 15.5. Divide the number of cells in the MN BNCs population by the number of cells in the BNCs population then multiply by 100:

$$\frac{MN\ BNCs}{BNCs} \times 100$$

16.2. Calculating cytotoxicity: Determine the total number of POLY cells by summing the number of TRI and QUAD cells.

16.2.1. Calculate the Cytokinesis-Block Proliferation Index (CBPI) by using the number of cells in the MONO, BNCs and POLY as follows:

$$CBPI = \frac{[(MONO) + (2 \times BNCs) + (3 \times POLY)]}{MONO + BNCs + POLY}$$

16.2.2. Finally, calculate the cytotoxicity of each culture by using the CBPI values from the control cultures (C) and chemically exposed culture (T) as follows:

$$Cytotoxicity = 100 - 100\left[\frac{(CBPI_T - 1)}{(CBPI_C - 1)}\right]$$

REPRESENTATIVE RESULTS

The analysis method outlined in this paper allows for the automatic identification and scoring of BNCs, with and without MN, to calculate genotoxicity. In addition, MONO and POLY cells are also automatically identified and scored to calculate cytotoxicity. Published scoring criteria[6,33] that must be adhered to when scoring these events are implemented in the MIFC data analysis software. The results presented here indicate that statistically significant increases in MN frequency with increasing cytotoxicity can be detected following exposure of human

lymphoblastoid TK6 cells to well-known MN inducing chemicals (mitomycin C and colchicine). Similar results for additional chemicals tested have been demonstrated in a separate publication[31]. In addition, results from the use of mannitol show that non-MN inducing chemicals can also be correctly identified using the MIFC method outlined here. The parameters described in the protocol to create all masks, features and region boundaries will likely have to be adjusted if different cell types (e.g. Chinese Hamster cells) are used to perform the assay.

Figure 3 shows four selected panels to identify BNCs (**Figure 3A-3D**). Also shown are bivariate plots that enable the selection of BNCs with similar circularity (**Figure 3B**), similar areas and intensities (**Figure 3C**) and BNCs that have well-separated, non-overlapping nuclei (**Figure 3D**) as per the scoring critieria[6,33]. **Figure 3E** shows the BF and Hoechst images as well as the BNC and MN masks indicating that BNCs with single or multiple MN can be identified and enumerated. This allows genotoxicity to be calculated by determining the rate of micronucleated BNCs in the final BNC population. **Figure 4** shows the application of the Spot Count feature using the POLY mask to identify MONO, TRI and QUAD cells. The number of TRI and QUAD cells can then be summed to obtain the final number of POLY cells (**Table 1**). This enables cytotoxicity to be calculated by using the formula shown in the protocol. Therefore, each dose point in the experiment can be evaluated by both genotoxicity and cytotoxicity parameters.

Figure 5 shows genotoxicity and cytotoxicity values for the aneugen colchicine, the clastogen mitomycin C and for a negative control, mannitol. For colchicine (**Figure 5A**) the 0.02 through 0.05 µg/mL doses produced statistically significant increases in MN frequency, ranging from 1.28% to 2.44% respectively over the solvent control (**Table 1**). In the case of the MMC (**Figure 5B**) the two top doses of 0.4 and 0.5 µg/mL produced statistically significant MN frequencies when compared to solvent controls. These MN frequencies were 0.93% at 0.4 µg/mL and 1.02% at 0.5 µg/mL (**Table 2**). Finally, for mannitol (**Figure 5C**), no doses tested induce a cytotoxicity over 30%, nor did they produce significant increases in MN frequency when compared to solvent controls, as expected (**Table 3**).

FIGURE AND TABLE LEGENDS

Figure 1: MIFC instrument settings. A screenshot of the MIFC settings as described in section step 7 of the protocol. (**A**) Setting the 405 nm laser power to 10 mW. (**B**) Setting the BF channels 1 and 9. (**C**) Selecting the 60x magnification objective lens. (**D**) Selecting the slowest flow speed which generates imagery with the highest resolution. (**E**) Specifying the number of events to be collected to 20,000. (**F**) Clicking the **Load** button to begin the sample load process. (**G**) Clicking the **Acquire** button to begin acquiring imagery. (**H**) Clicking the **Return** button to return any unused sample. (**I**) Scatterplot of BF Aspect Ratio versus BF Area for the selection of single cells. (**J**) Scatterplot of Hoechst Gradient RMS versus BF Gradient RMS for the selection of focused cells. (**K**) Histogram of Hoechst intensity for the selection of DNA positive cells.

Figure 2: Analysis software gating strategy. A screenshot of the gating strategy described in section 9 of the protocol. Regions are shown in sequential order for the identification of

binucleated cells (red box), micronuclei (yellow box), and mono- and polynucleated cells (blue box).

Figure 3: Identification and scoring of BNCs with and without MN. (**A**) Selection of cells that have two distinct nuclei. (**B**) Identification of binucleated cells (BNCs) that have two highly circular nuclei through the use of the Aspect Ratio Intensity feature. (**C**) Selection of BNCs that have nuclei with similar areas and intensities. This is accomplished by calculating the ratio of the area of both nuclei and the ratio of the aspect ratio of both nuclei. (**D**) Use of the Shape Ratio and Aspect Ratio features to identify BNCs that have two well-separated nuclei. (**E**) The Spot Count feature using the micronucleus (MN) mask demonstrating that BNCs with single or multiple MN can be identified and enumerated.

Figure 4: Identification and scoring of MONO and POLY cells. Use of the spot count feature to identify and enumerate mono-, tri- and quadranucleated cells. Component mask 1 allows the identification of mononucleated cells (top image). Component masks 1 through 3 allows the identification of trinucleated cells (middle image). Component masks 1 through 4 allows the identification of quadranucleated cells (bottom image). This figure has been modified from Rodrigues 2018[31].

Figure 5: **Quantification of cytotoxicity.** Cytotoxicity quantified using the cytokinesis block proliferation index (black circles) and genotoxicity quantified using the percentage of MN (clear bars) following a 3 h exposure and 24 h recovery for (**A**) colchicine, (**B**) mitomycin C and (**C**) mannitol. Statistically significant increases in MN frequency compared to controls are indicated by stars (chi-squared test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). All quantities are the average of two replicates at each dose point. This figure has been modified from Rodrigues 2018[31].

Table 1: The parameters required to calculate cytotoxicity (the number of mono-, bi- and polynucleated cells) and genotoxicity (the number and percentage of micronucleated binucleated cells) for colchicine. All calculated quantities are the average of two replicates at each dose point.

Table 2: The parameters required to calculate cytotoxicity (the number of mono-, bi- and polynucleated cells) and genotoxicity (the number and percentage of micronucleated binucleated cells) for mitomycin C. All calculated quantities are the average of two replicates at each dose point.

Table 3: The parameters required to calculate cytotoxicity (the number of mono-, bi- and polynucleated cells) and genotoxicity (the number and percentage of micronucleated binucleated cells) for mannitol. All calculated quantities are the average of two replicates at each dose point.

DISCUSSION:

In a recent publication Verma et al. underscored the importance of developing a system that combines the high-throughput advantage of flow cytometry with the data and image storage

benefits of image analysis[34]. The MIFC in vitro MN assay described in this paper satisfies this quotation and has the potential to overcome many of the aforementioned challenges in microscopy and flow cytometry methods. This protocol described here demonstrates that both cytotoxicity and genotoxicity can be evaluated using MIFC. Sample preparation, cellular staining and data collection are straightforward but there are some critical steps in the protocol that should always be implemented. Addition of potassium chloride (KCl) to the cells is critical to swell the cells, generating separation between the main nuclei and MN. This ensures that the masking algorithm can identify all individual nuclei in BNCs and POLY cells (POLY cells) which is necessary for their enumeration. Additionally, KCL provides separation between nuclei and MN, which is essential for accurate MN masking and quantitation. In addition, the addition of formalin following the addition of KCl prevents cells from lysing during centrifugation. The addition of cytochalasin B causes TK6 cells that have undergone more than one nuclear division to be quite large. As a result, the cytoplasm becomes fragile and can lyse if centrifugation is performed immediately after the addition of KCl. Moreover, it is very important to introduce Hoechst to the sample according to number of cells in the sample and not according to a final concentration. For example, a final concentration of 10 µg/mL of Hoechst will uniformly stain a sample of 1 x 10^6 cells but may not adequately stain a sample containing 5 x 10^6 cells and can result in many cells with dimly stained nuclei, making analysis difficult. It is also important to note that Hoechst can be replaced with another DNA dye such as DAPI if the MIFC is equipped with the 405 nm excitation laser or DRAQ5 if the MIFC is equipped with the 488 nm and/or 642nm excitation laser(s). If modifying the nuclear stain, it is critical to titrate the stain in order to find the appropriate concentration for the required/desired laser power.

When collecting data on the MIFC it is important to determine the optimal region boundaries for the Gradient RMS features. The boundaries presented in this protocol may require adjustment due to some slight variations between MIFC instruments. The application of this feature during data collection is essential to ensure that highly focused imagery is captured. If data files contain many blurred or unfocused images, it is probable that the masking algorithms in the analysis software will incorrectly highlight staining artifacts in the blurred areas, leading to a high number of false positive artifacts being scored as MN. Although the image processing techniques described here can be difficult, once an analysis template has been developed in the MIFC software, batch processing allows for data files to be automatically analyzed, eliminating user intervention and therefore, scorer bias. Also, if a cell line other than TK6 cells are used to perform the assay, it will be necessary to modify the masks and region boundaries as the morphological properties (e.g., size) of cells will differ from those of TK6 cells.

The results presented here (**Figure 5**) show statistically significant increases in MN induction when exposing TK6 cells to various doses of mitomycin C and colchicine. Statistically significant increases in the frequency of MN when compared to solvent controls were observed for several doses in both chemicals. In addition, no dose of mannitol induced a cytotoxicity over 30%, nor a statistically significant increase in the frequency of MN when compared to solvent controls, as expected. The protocol described in this paper using MIFC to perform the in vitro MN assay gives expected results from both positive and negative control chemicals. It is very important to perform a number of experiments using both solvent controls and negative control chemicals to

develop baseline values of both the frequency of MN as well as the Cytokinesis Block Proliferation Index (CBPI). For genotoxicity, statistically significant increases in MN frequency are determined through comparison to baseline MN frequencies which must be well-known for the cell type being used. In addition all cytotoxicity calculations are based on the CBPI of the control samples and therefore, baseline rates of MONO, BNCs and POLY cells must be well quantified in controls.

Several limitations and advantages of using MIFC in the context of the MN assay have been described in previous work[29,31]. The main limitations concern lower MN frequencies when compared to microscopy, which probably results from both the lack of flexibility when implementing the scoring criteria in the analysis software as well as the limited depth of field of the MIFC. Well-contoured masks can be created to accurately identify the main nuclei but MN that are touching (or very close to) the main nuclei might be captured within the BNC mask. Additionally, very small MN that can be rather easily scored using microscopy are probably incorrectly missed when using MIFC due the lower limit on the area parameter of the MN mask to avoid scoring small artifacts. In addition to the difficulties present in image-based data analysis, due to its design, MIFC obtains two dimensional projection images of three dimensional cellular objects. This likely causes some MN to be captured at a different depth of focus that the two main MN, making them appear very dim and un-scorable using masking. Moreover, a small fraction of MN could reside behind one of the two main nuclei, making them impossible to visualize and score. Therefore, considering these difficulties, caution should be used when interpreting significant increases in MN frequency at low doses.

Despite these shortcomings, the MIFC method described here offers several advantages over techniques. Fenech et al. proposed criteria and guidelines that should be considered when developing automated systems and methodologies for MN assays[35]. These include, but are not limited to, direct visualization of the main nuclei and cytoplasm, determination of the frequency of MN from various doses of the chemical or agent being tested and the ability to quantitate morphology and determine the position of all nuclei and MN to ensure they are within the cytoplasm. This paper shows that the MIFC method developed to perform the in vitro MN assay satisfies (or possesses the potential to satisfy) these criteria. Specifically, images of the nuclei and MN can be captured by the fluorescent lasers while cytoplasmic imagery can be obtained by using the BF LED. Imagery of cells with normal nuclear morphology can be automatically differentiated from those cells with irregular morphology using a combination of advanced masks and features. The results presented for colchicine and mitomycin C (**Figure 5**) show that both genotoxicity and cytotoxicity can be assessed at various doses when compared to solvent controls and that statistically significant MN frequencies are observed where expected. Furthermore, the OECD Test Guideline 487 recommends scoring 2,000 BNCs per test concentration to assess the presence of MN to determine genotoxicity along with at least 500 cells per test concentration to determine cytotoxicity[9]; this can take over 1 h using manual microscopy. The protocol and results in this paper show that an average of about 6,000 BNCs, 16,000 MONO cells, and 800 POLY cells were captured and scored per test concentration in about 20 min. The rapid rate of data acquisition and the high numbers of candidate cells scored in such a short time highlight another important advantage of employing MIFC to perform the in vitro MN assay.

While the results presented in this paper are encouraging, they are representative of an early proof-of-concept method. This work should be followed up by more thorough investigation of a larger, more diverse chemical set that covers multiple classes and mechanisms of genotoxicity and cytotoxicity such as those suggested by Kirkland et al.[36] Conducting such studies are time consuming and labor intensive, and fall outside of the scope of this paper however, these larger scale studies will provide valuable insight into the ability of the method to reliably identify weakly genotoxic agents. The methodology presented here has not yet been miniaturized to a microwell format, which would allow more rapid and efficient screening across a larger dose range. As such, in its current form, the MIFC-based in vitro MN assay presented here may be best suited for labor-intensive follow-up studies or research into good laboratory practices. However, the method will continue to be optimized and validated, and possesses the potential to allow for increased flexibility in detecting chemical specific events related to morphology, such as aneugen exposure that increases the proportion of cells with non-circular nuclei that are still scorable[37]. Finally, the MIFC method presents an opportunity to introduce additional biomarkers into the MN assay (e.g. kinetochore staining) to provide a more comprehensive view of the mechanism of MN induction.

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DISCLOSURES:

The author is employed by MilliporeSigma, the maker of the ImageStream multispectral imaging flow cytometer that was used in this work.

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Figure 1

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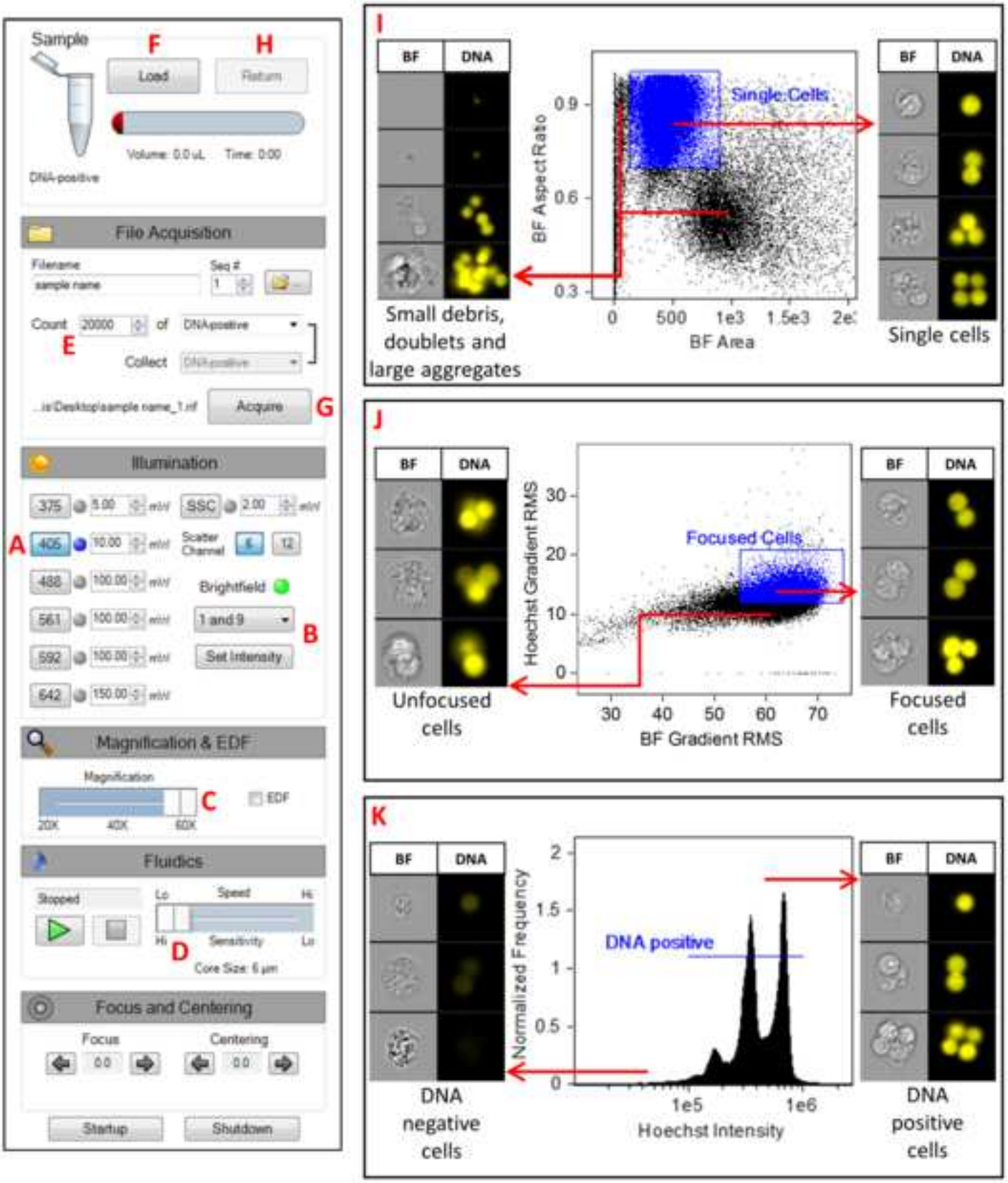

Figure 2

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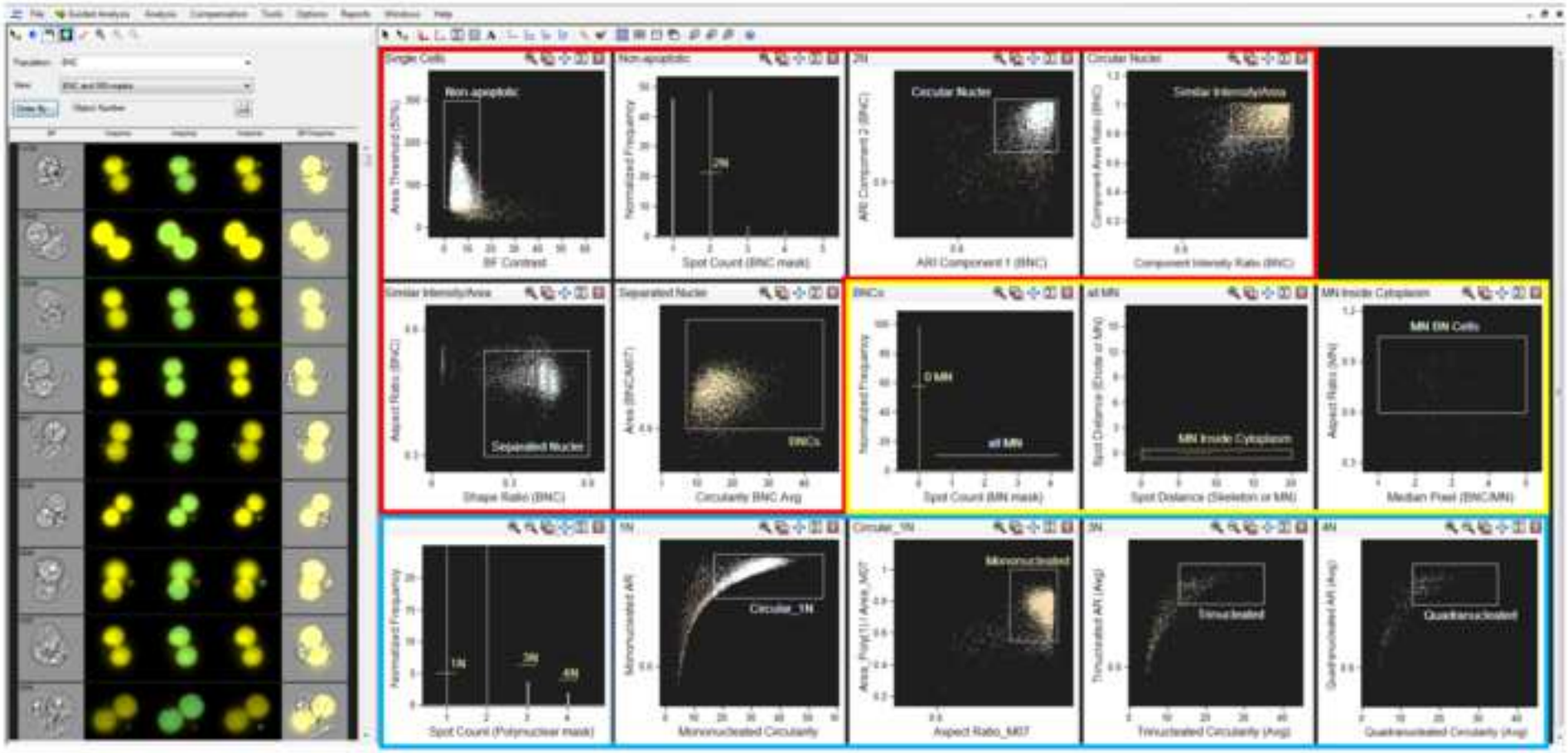


Figure 3

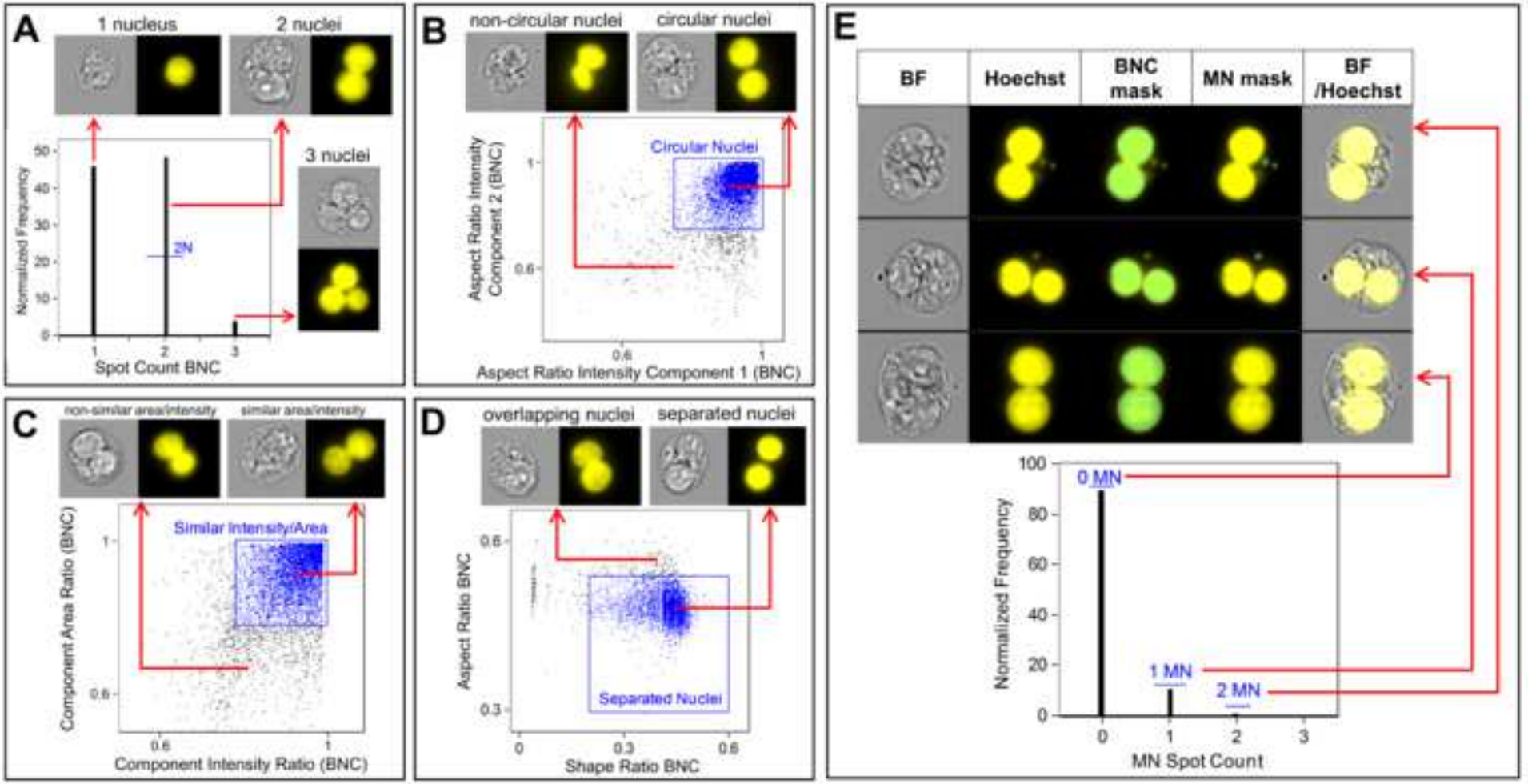


Figure 4

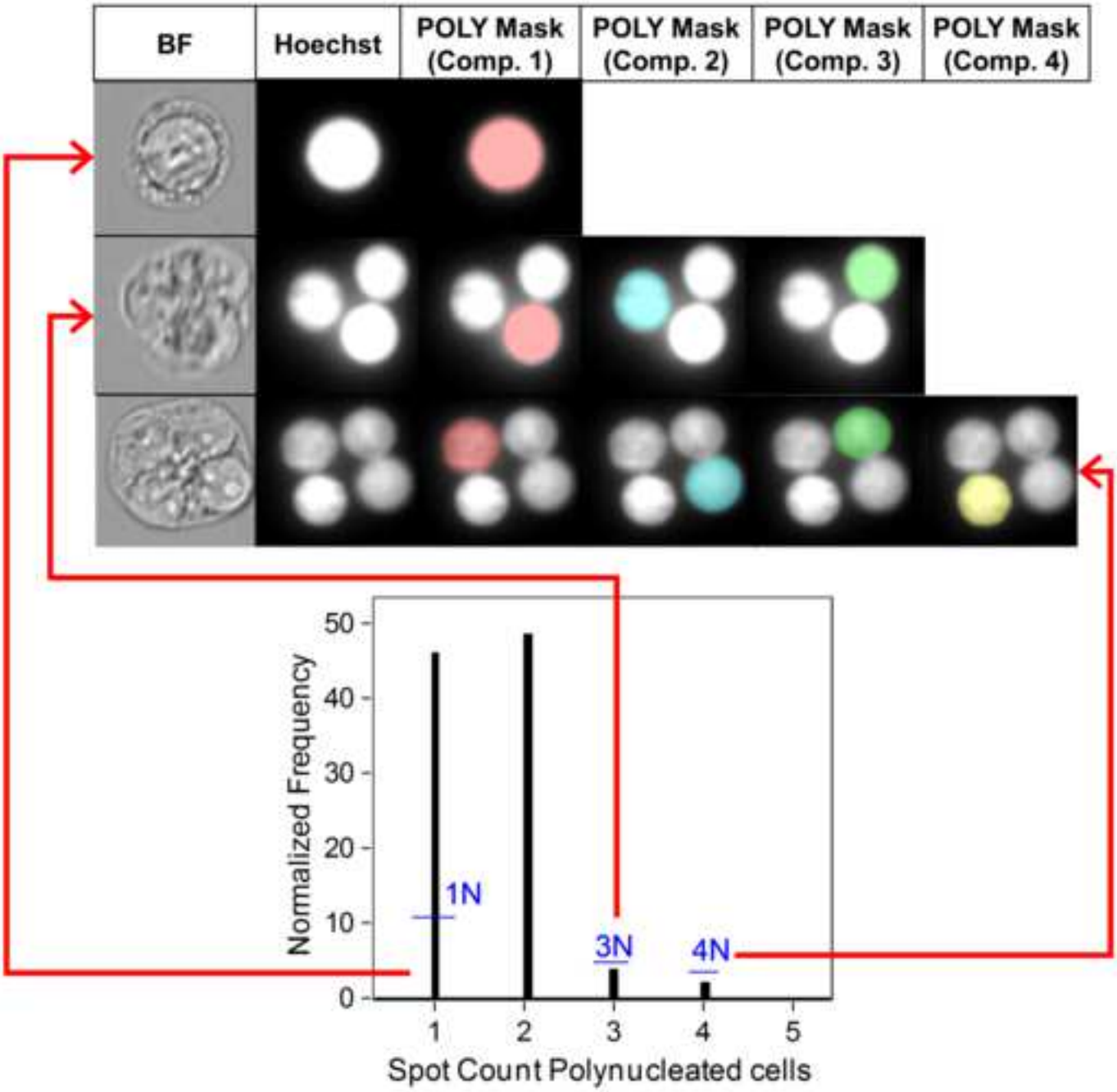
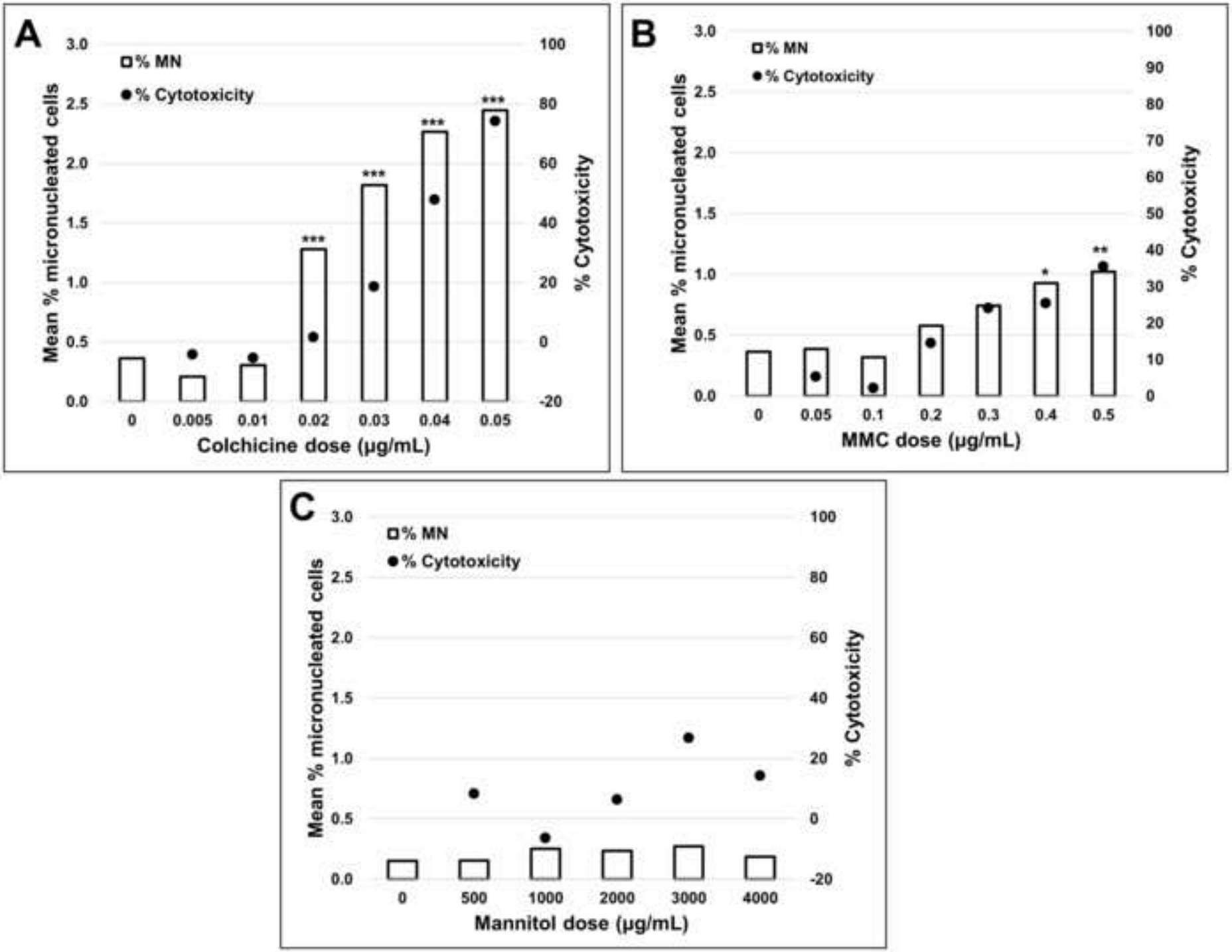


Figure 5



Colchicine

Dose (µg/mL)	Sample Number	Cytotoxicity		
		Number of Mononucleated cells	Number of Binucleated cells	Number of Polynucleated cells
0	1	6150	3167	1098
0	2	6628	3181	1091
0.005	1	6523	3135	1113
0.005	2	6038	3099	1344
0.01	1	5970	3381	1192
0.01	2	6104	3487	976
0.02	1	5242	4918	267
0.02	2	5679	4155	289
0.03	1	5475	3721	152
0.03	2	5883	3427	123
0.04	1	6765	2157	110
0.04	2	6816	2170	110
0.05	1	8846	1155	96
0.05	2	9110	1095	103

* statistically significant increase in MN ($p < 0.05$)

** statistically significant increase in MN ($p < 0.01$)

*** statistically significant increase in MN ($p < 0.001$)

	Genotoxicity	
% Cytotoxicity (Avg.)	Number of binucleated cells with micronuclei	% Micronucleated binucleated cells (Avg.)
---	11 12	0.36
-4.2	6 7	0.21
-5.5	12 9	0.31
1.5	65 51	1.28***
18.5	70 60	1.82***
47.7	57 41	2.26***
74.2	27 28	2.44***

Mitomycin C

Dose (µg/mL)	Sample Number	Cytotoxicity		
		Number of Mononucleated cells	Number of Binucleated cells	Number of Polynucleated cells
0	1	8342	2755	915
0	2	7802	3002	885
0.05	1	7235	3473	513
0.05	2	7890	2969	485
0.1	1	7938	3334	500
0.1	2	7273	3866	404
0.2	1	7732	3979	234
0.2	2	8680	3298	199
0.3	1	8834	3505	100
0.3	2	8819	3352	186
0.4	1	8599	3783	115
0.4	2	9006	2992	123
0.5	1	9852	3018	90
0.5	2	9473	3149	99

* statistically significant increase in MN ($p < 0.05$)

** statistically significant increase in MN ($p < 0.01$)

*** statistically significant increase in MN ($p < 0.001$)

	Genotoxicity	
% Cytotoxicity (Avg.)	Number of binucleated cells with micronuclei	% Micronucleated binucleated cells (Avg.)
---	10 11	0.36
5.3	14 11	0.39
2.1	11 12	0.32
14.5	18 24	0.58
24.1	25 26	0.74
25.4	35 28	0.93*
35.4	29 34	1.02**

Mannitol

Dose (µg/mL)	Sample Number	Cytotoxicity		
		Number of Mononucleated cells	Number of Binucleated cells	Number of Polynucleated cells
0	1	8278	2474	485
0	2	8511	2833	518
500	1	8584	2652	377
500	2	8868	2565	422
1000	1	7759	3168	533
1000	2	8397	2754	378
2000	1	8691	2458	424
2000	2	8678	2679	480
3000	1	9640	2512	241
3000	2	9804	2242	245
4000	1	9397	2673	288
4000	2	9185	2738	350

* statistically significant increase in MN ($p < 0.05$)

** statistically significant increase in MN ($p < 0.01$)

*** statistically significant increase in MN ($p < 0.001$)

	Genotoxicity	
% Cytotoxicity (Avg.)	Number of binucleated cells with micronuclei	% Micronucleated binucleated cells (Avg.)
---	35	0.15
8.3	35	0.15
-6.4	87	0.25
6.3	75	0.23
26.7	67	0.27
14.2	73	0.18

Table of Materials

Click here to access/download;Table of Materials;Specific Materials and Equipment-v1-0.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
15 mL centrifuge tube	Falcon	352096	
Cleanser - Coulter Clenz	Beckman Coulter	8546931	Fill container with 200 mL of Cleanser. https://www.beckmancoulter.com/wsrportal/page/itemDetails?itemNumber=8546931#2/10//0/25/1/0/asc/2/8546931///0/1//0/
Colchicine	MilliporeSigma	64-86-8	
Corning bottle-top vacuum filter	MilliporeSigma	CLS430769	0.22 um filter, 500 mL bottle
Cytochalasin B	MilliporeSigma	14930-96-2	5 mg bottle
Debubbler - 70% Isopropanol	EMD Millipore	1.3704	Fill container with 200 mL of Debubbler. http://www.emdmillipore.com/US/en/product/2-Propanol-70%25-%28V%2FV%29-0.1-%C2%B5m-filtred,MDA_CHEM-137040?ReferrerURL=https%3A%2F%2Fwww.google.com%2F
Dimethyl Sulfoxide (DMSO)	MilliporeSigma	67-68-5	
Dulbecco's Phosphate Buffered Saline 1X	EMD Millipore	BSS-1006-B	PBS Ca++MG++ Free
Fetal Bovine Serum	HyClone	SH30071.03	
Formaldehyde, 10%, methanol free, Ultra Pure	Polysciences, Inc.	04018	This is what is used for the 4% and 1% Formalin. CAUTION: Formalin/Formaldehyde toxic by inhalation and if swallowed. Irritating to the eyes, respiratory systems and skin. May cause sensitization by inhalation or skin contact. Risk of serious damage to eyes. Potential cancer hazard. http://www.polysciences.com/default/catalog-products/life-sciences/histology-microscopy/fixatives/formaldehydes/formaldehyde-10-methanol-free-pure/
Hoechst 33342	Thermo Fisher	H3570	10 mg/mL solution
Mannitol	MilliporeSigma	69-65-8	
MEM Non-Essential Amino Acids 100X	HyClone	SH30238.01	
MIFC - ImageStreamX Mark II	EMD Millipore	100220	A 2 camera ImageStreamX Mark II eqiped with the 405nm, 488nm, and 642nm lasers was used. http://www.emdmillipore.com/US/en/life-science-research/cell-analysis/amnis-imaging-flow-cytometers/imagestreamx-Mark-ii-imaging-flow-cytometer/VaSb.qB.QokAAAFLzRop.zHe,nav?cid=BI-XX-BDS-P-GOOG-FLOW-B325-0006
MIFC analysis software - IDEAS	EMD Millipore	100220	The companion software to the MIFC (ImageStreamX MKII)
MIFC software - INSPIRE	EMD Millipore	100220	This is the software that runs the MIFC (ImageStreamX MKII)
Mitomycin C	MilliporeSigma	50-07-7	
NEAA Mixture 100X	Lonza BioWhittaker	13-114E	
Penicllin/Streptomycin/Glutamine solution 100X	Gibco	15070063	
Potassium Chloride (KCl)	MilliporeSigma	P9541	
Rinse - Ultrapure water or deionized water	NA	NA	You can use any ultrapure water or deionized water. Fill container with 900 mL of Rinse.
RNase	MilliporeSigma	9001-99-4	
RPMI-1640 Medium 1X	HyClone	SH30027.01	
Sheath - PBS	EMD Millipore	BSS-1006-B	This is the same as Dulbecco's Phosphate Buffered Saline 1X Ca++MG++ free. Fill container with 900mL of Sheath.
Sterile water	HyClone	SH30529.01	
Sterilizer - 0.4-0.7% Hypochlorite	VWR	JT9416-1	This is assentually 10% Clorox bleach that can be made by deluting Clorox bleach with water. Fill container with 200 mL of Sterilzer.
System Calibration Reagent - SpeedBead	EMD Millipore	400041	Each tube holds ~10 mL. https://www.emdmillipore.com/US/en/life-science-research/cell-analysis/amnis-imaging-flow-cytometers/support-training/XDqb.qB.wQMAAAFLBDUp.zHu,nav
T25 flask	Falcon	353109	
T75 flask	Falcon	353136	
TK6 cells	MilliporeSigma	95111735	

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Author(s): Matthew A. Rodrigues

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Article Title:	Automation of the In Vitro Micronucleus Assay using Multispectral Imaging Flow Cytometry
Signature:	 Date: 10/30/2018

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I would like to thank the editors and reviewers for their thorough review of my manuscript. There are several constructive comments here, which with their incorporation, will enhance the quality of the manuscript.

Below are my responses to all individual comments and action items. For comments that required editing the manuscript I have done so and highlighted the new content in red in the manuscript. In this rebuttal letter I have also included any text that has been added to the manuscript so that you don't have to search through the manuscript to find details. However, should you wish to read the new text within the manuscript, I've also included updated line numbers.

I feel that the modifications made to the manuscript now satisfy all of the requests from the editors and reviewers required for publication.

Editorial comments

Reviewer #1 has expressed some concern at the length of the protocol and some seemingly trivial details within (see minor comment #2). Before I make any changes to the protocol, I'd like to ask that you review it to see if you'd like it to be shortened in the main body of the manuscript.

Reviewer #2 has expressed some concern regarding the existence of this manuscript since the results presented here are very similar to a recent paper that I published in Cytometry Part A. I'd ask that you review the comment (see major comment #2) as well as my rebuttal when considering whether to accept this manuscript.

Changes to be made by the author(s) regarding the manuscript:

1. **Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**

   I have proofread the manuscript and run spellchecker in Word. To the best of my knowledge there are no grammar or spelling errors.

2. **Lines 660-663: Please rephrase to avoid direct quotation.**

   I feel that the quotation from the Verma article is rather eloquent and highlights a rather important gap in this field, which is why I've quoted the text directly. Nevertheless, I've changed the sentence so that it is no longer a direct quote. The sentence now reads (lines 666-668):

   In a recent publication Verma et al. underscored the importance of developing a system that combines the high-throughput advantage of flow cytometry with the data and image storage benefits of image analysis.

3. **JoVE article does not have an Abbreviations section. Please remove lines 20-28 and define all abbreviations before use**

   I have removed the abbreviations section. Going forward, you might consider allowing flexibility in your formatting to include abbreviations sections for longer manuscripts as they are quite helpful to readers

4. **Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).**

   After reviewing the manuscript, the only instance I could find with personal pronouns was the word “your” in section 14.4. I have changed this to “the”

5. **Please change the time unit “hr” to “h”.**

   I have made this change throughout the manuscript

6. **3.1: Please specify the chemical used in this step.**

   The chemical used here can be any water soluble chemical which is why I did not indicate a specific chemical. However, I have added (e.g. Mitomycin C) after the words “desired chemical”

7. **3.3: Please specify the recovery time used.**

   As with point 6, I wanted to leave this more general since the doubling times for all cell types are slightly different, which is why I indicated the guidance from the OECD to use a recovery time of 1.5-2.0 doubling times. However, to address this comment I have added this line to point 3.3: *For the TK6 cells used in this work, the recovery time was 24 h.*

8. **Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.**

   I have made sure that, for the most part, individual do not contain more than 3 actions. However, in a few cases it is necessary to have more than 3 actions in a single step to avoid the instructions from being unnecessarily fragmented.
   Examples of this include step 7 that describes how to set up the MIFC, create plots and draw regions. Breaking up steps 7.2 and 7.3, for example, into sub steps will just confuse the reader. In addition, some complicated steps in the data analysis template creation portion of the protocol have more than 3 actions per step. Step 10.1.1.1, for example, has 8-10 actions (depending on what you define as an action). It makes sense in the context of this step to have all of these actions as one step. Fragmenting these steps will make things very confusing for the user.

9. **Please ensure that the highlighted portion identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.**

   This is a complicated protocol in which many steps have multiple sub steps, especially in the data analysis portion of the protocol. Some steps that have been highlighted (e.g. 9.3) contain many sub steps and highlighting all of these steps will make the highlighted portion far exceed the required 2.5 pages. Because of this, I uploaded a document called “Notes for Filming of Protocol” with the submission for you to consult and review.

In this document I outline my recommendations for filming these sections that do not have every sub step highlighted since the method presented in this manuscript is complex (especially the data analysis).
In my view, there are two options:
1. I can highlight all sub steps which will make the highlighted portion go well beyond 2.5 pages.
2. Leave the highlighted sections as they are and work with you on a filming protocol that will cover the key portions of each step.
I prefer option 2 since I have a vision for how to film each of these complicated steps. This probably deviates from what you are used to, but it may just have to be that way for this manuscript.

10. Table of Equipment and Materials: Please sort the items in alphabetical order according to the name of material/equipment.

This has been done.

11. References: Please do not abbreviate journal titles.

Sorry about that, I've fixed this. I was looking at older JoVE publications (e.g. before 2018) and those all have abbreviated journal names. Also, you should be aware that the most currently available Endnote library default style that I am using for references (available from the Endnote website) is to abbreviate journal names. You may want to contact Endnote to have them update the style since many researchers use this software.

Reviewer #1 comments

Major Concerns:
None.

Minor Concerns:

1. **Clarity is required over the number of chemical analysed in this paper. The results presented show the method tested for Mitomycin C, Colchicine, and Mannitol. The abstract, introduction and results sections suggest that results demonstrate significant MN frequencies following exposure to known aneugens and clastogens. The use of plurals is misleading suggesting more chemicals have been tested than 2 known positives and 1 known negative. It would be less misleading to name the chemicals tested within the abstract Line 57 and the representative results Lines 576-577, whilst referring to other studies which show results for further chemicals.**

This is a good point. This manuscript is a condensed version of a recent paper that I published in Cytometry Part A (93, 706-726, 2018). In that paper I examined 4 well-known MN inducers and Mannitol as a negative control. In the interest of providing condensed results in this manuscript I chose to show results for only two chemicals used in that paper (Mitomycin C and Colchicine) as well as Mannitol to demonstrate that non-MN inducing chemicals can also be correctly identified. To reflect this, I have changed the last sentence in the abstract to read (lines 44-48):

Results demonstrate that using MIFC to perform the in vitro MN assay allows statistically significant increases in MN frequency to be detected at several different levels of cytotoxicity when compared to solvent controls following exposure of TK6 cells to Mitomycin C and

Colchicine, and that no significant increases in MN frequency are observed following exposure to Mannitol.

I have also changed the text in the results section to read (lines 576-581):
The results presented here indicate that statistically significant increases in MN frequency with increasing cytotoxicity can be detected following exposure of human lymphoblastoid TK6 cells to well-known MN inducing chemicals (Mitomycin C and Colchicine). Similar results for additional chemicals tested have been demonstrated in a separate publication31. In addition, results from the use of Mannitol show that non-MN inducing chemicals can also be correctly identified using the MIFC method outlined here.

2. **I agree that a detailed SOP is useful for the reader and a valuable guide to reproduce the technique but feel that a much more summarised method should be included in the materials and methods section of the paper with the absolute finer details incorporated into the supplementary section. For example, details of how to prepare each chemical used in the methods such as how to prepare 10 mL of 4% formalin, is not required by the reader.**

   In principle I agree with you on the fact that including these rather trivial steps is not necessary. However, I have seen a number of JoVE papers that have very detailed protocols in the main text, containing similarly simple details. Since you have listed this as a minor concern, I will leave the protocol as is and request that the editor(s) review the protocol with a critical eye. If they wish for such small details to be removed from the main and into the supplementary material, I'll be happy to make this change.

3. **Similarly, the number of Notes present in the methods is excessive. One Note at the beginning or end of the SOP is sufficient or Note points should be incorporated into the supplementary materials.**

   I have put one note of caution for Cytochalasin B and Formalin/formaldehyde at the beginning of the protocol and have incorporated others into sub steps. Now there are only three notes in the protocol and, in my view, it is important that they stay where they are to ensure that the method is performed correctly.

4. **The methodology used to produce the representative results is not stated clearly. For example in point 2. of the Protocol Lines 147-163, a separate section should be included stating the actual methods used (i.e. the chemicals utilised and dose ranges) to produce the results in Figure 5 and Tables 1, 2, and 3. Reference to the table of materials needs to be included early on the protocol.**

   I have expanded point 2.3 to include the specific doses used to produce the results in Figure 5. That point now reads (lines 163-166):

   For Mitomycin C, prepare 3 mL dilutions in sterile water of 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 µg/mL. For Colchicine, prepare 3 mL dilutions in sterile water of 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 µg/mL. Finally, for Mannitol, prepare 3 mL dilutions in sterile water of 5, 10, 20, 30, 40 and 50 mg/mL.

   References to the table of materials has been added to points 1.1, 1.2, 2.1 and 4.

5. **It would be helpful to offer more images of example scatter plots observed during MIFC or refer to the relevant Figures (2, 3 and 4) more in the material and methods. For example in section 7.3 Line 269 a reference to Figure 2 would be useful as a guide to the population of cells that needs to be gated.**

   This is a good point. Although there is a very detailed figure explaining this in my recent Cytometry A paper (2018) which is open access, having a similar figure here could help users acquire data correctly.
   I have expanded Figure 1 to include the three plots that should be created during data acquisition and have included reference imagery of what events should and should not be collected. I have referenced this figure where appropriate in section 7 of the protocol.

6. **There appears to be some self-citation occurring (Refs 26-30).**

   There is a lot of self-citation occurring (refs 26-32 actually). Despite five years of research and multiple publications on this topic, the MIFC and the method presented in this manuscript will be new to most readers. Therefore, in my view, it is critical that readers are aware of the additional papers that have been published on this work (most of which are open access) since they will no doubt want more information on the protocol, results, chemicals tested, and additional fields of study where the method has been applied.

Reviewer #2:

Major Concerns:

1. **The author raises several advantages of the method that are brought about by the incorporation of the imaging cytometer. Do any of these address/solve specific regulatory-related issues that other methods struggle with or are they simply characteristics of the instrument/workflow that lend some benefits to certain use cases? In other words it's not as if other existing methods are unable to achieve certain parameters stated by regulatory agencies and your system has overcome them, correct? I raise this not so much as a knock against your approach, rather as recognition that your system arguably has a high bar for entry in terms of instrument cost, operator skill, etc and that should be balanced out against what advantages it really brings to the table.**

   Further, as stated your method is not miniaturized to a microwell format which would enable faster, more efficient screening, so perhaps it's more likely to be used in follow-up work, GLP studies or those that use more labor-intensive models such as cultured human lymphocytes. These considerations factor into the overall positioning of your assay and it's potential impact on workflow, which again is connected to cost-benefit considerations. It may be worth addressing this to some extent in your introduction or discussion so the reader can be clearly informed as to the most accurate positioning of this approach in their pipeline.

   At this point our method does not overcome any limitations regarding regulatory requirements that other systems struggle with. However, in the context of the requirements of the MN assay, the limitations of other systems are well described (e.g. lack of cytoplasmic visualization with Metafer and lack of any visualization with conventional flow). This may eventually lead to potential limitations or complications within the regulatory guideline framework when using those systems. For example, in a recent publication, Verma *et al*. (2017, Archives of Toxicology, 90: 2689-2698) demonstrated that automated slide scanning

(e.g. Metafer) and conventional flow cytometry either under- or overestimate MN frequencies when compared to manual microscopy using MMS and Carbendazim (the former is in the list of compounds in the paper you point out in major concern #3 (Kirkland *et al*. 2016, Mut. Res. Genet. Toxicol. Env. Mutagen. 795:7-30). So, it may be possible that other methods do indeed have limitations in the context of regulatory requirements that the community is not yet aware of, and more studies such as the one performed by Verma *et al*. are probably necessary to identify said limitations.

With respect to our system, we have not yet done enough validation work to determine whether we can address such limitations. To determine this, it will be necessary to validate the method against manual microscopy, automated microscopy as well as conventional flow cytometry. In my opinion, manual microscopy as a gold standard is inherently flawed and several difficulties have been pointed out in the literature, such as a limit on throughput which reduces the dose range that can be reliably tested and significant variability in results between scorers (Fenech *et al.*, 2003, Mutation Research, 534: 45-64; Lovell, *et al*., Mutat Res Gen Tox En, 825: 40-50, 2018). Large-scale validation studies with many samples using our system will allow us to examine reproducibility of results in both negative controls and samples exposed to chemicals, as well as investigating a wider range of doses than is feasible with manual microscopy.

With respect to cost, the ImageStream is obviously much more expensive than many fluorescent microscopes. However, with increasing interest in automating scoring, automated slide scanners such as the Metafer system offered by Metasystems are desirable. The cost of the Metafer system and the base model of the ImageStream are very similar so I do not see this as a deterrent. You are correct regarding the operator skill required to perform the current data analysis required with the ImageStream method, which is a disadvantage of this method but underscores the importance of this JoVE protocol manuscript. It is important to mention that some recent publications have demonstrated feasibility of machine learning algorithms to analyze ImageStream data (Hennig *et al*. Methods, 112: 201-210, 2017; Elunber *et al*. Nature Communications, 8, 463, 2017 ; Blasi *et al*. Nature Communications, 7: 10256, 2016). I envision that a machine learning algorithm could eliminate the need for the advanced IDEAS-based analysis presented in this manuscript, but until that is developed, this is the current best practice to analyze this data.

At the moment, no, our method is not miniaturized since this work is in the proof-of-concept stages and those protocols/optimizations will take time to develop. For example, as far as I can tell from the conventional flow cytometry method to perform the in vitro MN assay, four years passed between the first proof-of-concept studies and the first paper on miniaturization. Your points about where this method would fit into the workflow are interesting and I feel like the analytical abilities of the system could certainly enhance a number of aspects of the MN assay in general (and other cytogenetic assays). I have used this comment and attempted to address your concerns as part of the new closing paragraph of the discussion (lines 762-770):

The methodology presented here has not yet been miniaturized to a microwell format which would allow more rapid and efficient screening across a larger dose range. As such, in its current form, the MIFC-based in vitro MN assay presented here may be best suited for labor-intensive follow-up studies or research into good laboratory practices. However, the method will continue to be optimized and validated, and possesses the potential to allow for increased flexibility in detecting chemical specific events related to morphology, such as aneugen exposure that increases the proportion of cells with non-circular nuclei that are still

scorable[37]. Finally, the MIFC method presents an opportunity to introduce additional biomarkers into the MN assay (e.g. kinetochore staining) to provide a more comprehensive view of the mechanism of MN induction.

In my opinion, what our method brings to the table over other methods is quite substantial even at this very early, proof of concept stage. The ability to identify and quantify all key events required for genotoxicity/cytotoxicity simultaneously through automated data analysis is an obvious advantage over manual scoring. This is also an improvement over slide-based automated scoring in which multiple scans, using multiple classifier sets, are often required to identify all events. Moreover, the advantages over conventional flow cytometry where no image data is obtained at all are obvious (to me, the existence of a conventional flow method for the MN assay has always been an odd paradox in light of the number of publications and guidelines that place high importance on the morphology of the cells and being able to verify the legitimacy of supposed MN).

2. **As part of information gathering for this review, I see that the author recently published a very similar article in Cytometry A 2018. While I can potentially rationalize the existence of these two papers based on the unique video format of JOVE, the considerable overlap between these two works is worth considering. Perhaps the best example of this is found in Figure 5. These histograms appear to be exact copies of the ones already published in the Cytometry A paper (Figure 6). Is the author concerned about any potential copyright issues based on the presumed ownership of those images by the publisher of Cytometry A? What other overlap(s) should be considered as part of the review and approval process initiated by JOVE?**

   When I was approached by JoVE about writing this manuscript I was very clear with the editor I spoke with (Dr. Nandita Singh) that I would not have any new data/results to write about within the tight timeline proposed for submission. I also stated that if I were to write a manuscript for JoVE, it would feel very much like the 2018 Cytometry A paper you mention but that it would focus heavily on the data analysis portion of this work. Dr. Singh did not express any concerns about this and we both envisioned a manuscript that would be complementary to the Cytometry A paper, in which a step-by-step protocol to perform the assay would be described. I feel like I have written a manuscript that is very much in line with what Dr. Singh and I spoke of.

   Your concern is certainly valid, as was mine when I began to write the manuscript. However, I feel that since the manuscript focuses on working readers through the collection of MIFC data and the creation of an analysis method, the two papers can exist as individual entities without concern about overlap.

   Regarding Figure 6 (and any other content) from the Cytometry A article, appropriate written permissions have been obtained by Wiley to use the content in this JoVE manuscript, so this is not of concern. In addition, I corrected some overlap that appeared between this article and the Cytometry A article at the request of the editors following initial submission and prior to the article advancing to the peer review stage.

   I will leave it to the editors to review your comment and my rebuttal, and to decide whether to continue with publication of the manuscript.

3. **One current issue that is being addressed by the genetic toxicology community is the potential for the in vitro micronucleus test to generate false or irrelevant positive**

compound calls. As part of efforts to address this, there are published lists of compounds that can be used to demonstrate lab proficiency/method validity - See Kirkland et al 2016 Mut. Res. Genet. Toxicol. Env. Mutagen. 795: 7-30, also Table 1 in OECD 487 (2016). The author's choice of chemicals is okay in terms of positive agents (although a larger, more diverse set would be that much more convincing for the method's performance), however mannitol is hardly a challenging compound and does little to comment on the specificity of your system. Compounds that elicit significant toxicity/apoptosis and/or disrupt other essential cellular functions, aside from those associated with DNA, can give a much better indication of the overall ability of a method to accurately distinguish genotoxicants from non-genotoxic cytotoxicants. I acknowledge the additional effort it takes to conduct such studies and if they are deemed beyond the scope of this work, you might consider stating as such and cast the current investigation as an early proof-of-concept study that should be followed up by more extensive investigation of a larger, more diverse chemical set that covers multiple classes/mechanisms of genotoxicity and cytotoxicity.
This would also hopefully give you the opportunity to address another issue you raise with your method on line 726. Advising caution in the interpretation of results at low doses could also be seen as lack of ability to clearly identify agents with weak genotoxicity. It's often a simple matter to identify highly potent genotoxic compounds like MMC, but just as important to do the same with less potent examples such as propane sultone or 4NQO. You should consider addressing this as part of your discussion around the performance of your system at low doses.

The two chemicals used here (Mitomycin C and Colchicine), as well as the two additional chemicals used in the Cytometry A 2018 paper (MMS and Vinblastine Sulfate) are all recommended for use in Table 1 of the OECD guideline. They also all appear in the Kirkland *et al*. publication you mention (as does Mannitol). Using these documents as reference is actually how I chose to use these chemicals to perform experiments for the Cytometry A paper. I do agree that Mitomycin C and Colchicine are relatively simple to use and to observe a statistically significant increase in MN frequency, which is exactly why I used them. In my view, the first matter of importance was to ensure the method could detect MN frequency increases with strong genotoxicants. The natural next step is to investigate more compounds that appear in the Kirkland *et al*. publication that include weaker genotoxicants.

Regarding Mannitol, I actually feel that its use (and the results obtained) are highly important and I respectfully disagree with your assessment. In my opinion, results from a well-known negative control are imperative to demonstrate that the system does not detect false positive MN, and therefore does not generate false positive results. It is very important that the results presented here for Mannitol remain in the paper as they demonstrate that no significant MN frequencies are detected at any of the tested doses. This is just as important as being able to detect increases in MN frequencies with well-known genotoxicants.

Regarding larger scale studies with many more test chemicals, I agree entirely that we must perform such studies. However, as you point out, these studies are time consuming and require much effort to conduct, so it will certainly take quite a bit of time to perform the experiments and compile and interpret the results, so I do feel this extra work is outside of the scope of this paper, as you mention. But, this type of investigation will be an absolute requirement if this method is to be adopted for use by the wider toxicology community. To address this point, I have added the following text to the discussion as part of a new closing paragraph (lines 756-762):

While the results presented in this paper are encouraging, they are representative of an early proof-of-concept method. This work should be followed up by more thorough investigation of a larger, more diverse chemical set that covers multiple classes and mechanisms of genotoxicity and cytotoxicity such as those suggested by Kirkland et al.[36]. Conducting such studies are time consuming and labor intensive, and fall outside of the scope of this paper however, these larger scale studies will provide valuable insight into the ability of the method to reliably identify weakly genotoxic agents.

Minor Concerns

1. **Pg 2, ln 62 - The phrase "a wide variety of various environmental…" is awkward. Replace one use of various with an alternate word, perhaps "diverse"?**

   You're right, this is an awkward phrase. I've just removed the words "a wide variety of various" and now the sentence reads (lines 53-54):

   …among individuals exposed to environmental, occupational or lifestyle factors

2. **Pg 2, ln 64 - Arguably the chromosome fragments aren't generated specifically during metaphase/anaphase transition. They can occur at other points during the cell cycle and are simply revealed as a consequence of the separation of paired chromosomes.**

   This is true. However, the explanation of where in the cell cycle that MN are generated varies quite a bit in the literature. I've removed the specific mention of stages of the cell cycle and made this sentence more general (lines 54-56):

   MN consist of chromosome fragments or whole chromosomes generated during cell division that are not incorporated into one of the two main daughter nuclei.

3. **Pg 2, ln 68 - Micronuclei are often used as an indicator of chromosomal damage, either structural or numerical. They are considered relatively gross effects. Mutation has more to do with actual disruption of the gene sequence through base pair substitution, frameshifts, indels, etc. Depending on fidelity of repair and/or initiation of death there may or may not be actual negative consequences to gene sequence, e.g. fixed mutation subsequent to chromosomal damage, but it is not guaranteed. In my estimation it would be inaccurate to refer directly to micronuclei as representing gene mutation. I do recognize that your statement comes directly from an existing paper authored by genetic toxicologists, however I encourage you to consider my points and leave it up to your judgement to keep this statement in your manuscript.**

   You bring up a good point. The process that leads to expression (or not) of chromosomal damage is certainly complex and addressing that complexity within the introduction of this manuscript is probably outside of its scope. As such, I've changed the sentence you point out to be a bit more general (lines 57-59):

   Therefore, MN are representative of DNA damage and have been used for many years as an endpoint in genotoxicity testing.

4. **Pg 2, ln 78 - Regarding the lack of visualization of cytoplasm, what about methods that use metachromatic fluorescent dyes like acridine orange that stain the cytoplasm**

red and nuclear material green? Such methods can be used to clearly identify cytoplasm and determine the integrity of the cells being interrogated.

To the best of my knowledge alcidine orange (AO) is currently not compatible with automated slide scanning (e.g. Metafer). Every study I've read in which slides are stained with AO, they are only scored by visual microscopy and the slides scored by automated methods are nearly always stained with DAPI. This has to do with the classifier settings that are required for the Metafer system to perform properly, a limitation that has been mentioned in the literature (Verma *et al*., 2017, Archives of Toxicology, 90: 2689-2698).

So yes, the cytoplasm can be visualized when scoring slides manually, but not with the automated scoring techniques that I am mentioning in this sentence.

5. **Pg 2, ln 79 - This is a circular argument. In vitro micronucleus methods exist that don't require the use of cytochalsin B and these include mechanisms for the determination of cytotoxicity. The only reason you need to identify polynucleated cells in the in vitro MN method is to enable application of CPBI cytotoxicity metric which is a consequence of using cytochalsin B pushing some fraction of cells into a polyploid state. Please re-word or eliminate this statement.**

   You are correct about why polynucleated cells need to be identified in the Cyt-B version of the assay, but the sentence is not a circular argument. The sentence simply points out that by not being able to identify polynucleated cells, cytotoxicity cannot be determined when using Cyt-B. In addition, it has been pointed out in the literature that the non-Cyt-B versions of the assay may overestimate or underestimate MN frequencies and thus give false positive or negative results (e.g. Fenech, 2000, Mutagenesis, 15: 329-336 and Fenech, 2006, Mutation Research, 607: 9-12)

   However, I acknowledge that the sentence is somewhat poorly written, so I have rephrased it (lines 70-72):

   Furthermore, these methods have difficulties identifying polynucleated (POLY) cells (including tri- and quadranucleated cells) which are required for the calculation of cytotoxicity when using Cyt-B[9]*.*

6. **Pg 2, ln 86 - Similar to above, the regulations specify for which methods it is appropriate to employ cytochalsin B, but it is not required for every established methodology, especially those using immortalized cell lines. Reconsider using lack of ability to use cyt b as a disadvantage of alternative methods.**

   As with my response to concern #5, you're correct that not all methods use Cyt-B. However, as I point out above, evidence has been presented that false positive or negative results may be obtained without its use. In addition, many papers in the literature quantify MN frequencies from chemicals with and without Cyt-B, including several papers in a special edition of Mutation Research in 2010 dedicated to the in vitro MN assay (702 - In Vitro MN Trial: Cytotoxicity Measures in the In Vitro Micronucleus Test).

   Therefore, I would like to leave the lack of ability to use Cyt-B as a disadvantage of other methods in the manuscript.

7. **Pg 3, ln 123 - You use the word "simple" to describe the method, but what follows is a protocol description that includes 16 steps each with numerous sub-operations and the "Full Protocol" in the Supplementary files adds even more steps. Simple may not be the operative term, in fact you state later on (Pg 16, ln 693) that the "techniques described here can be difficult". Please rectify this inconsistency.**

   This is true. What I meant was that the wet lab portion of the protocol is simple in comparison to other techniques. You are right that the data analysis portion is quite difficult, hence the need for this manuscript.

   I've removed the words "simple yet elegant" from the sentence you referred to.

8. **Pg 6, ln 243 - Presumably some portion(s) of sections 6 onward are required for initial set-up, but can then simply be called-up for subsequent analytical runs, similar to loading an analysis template in other flow cytometers? If this is the case it would be wise to mention this so that the reader doesn't assume that all the steps laid out in the Full Protocol are required for every time the machine is turned on and samples are ready for analysis. On a related note it would arguably be useful to include some information on time/effort required to perform the various aspects of the method. How much time does it take for the benchtop processing for a typical study? What about analysis? These metrics speak again to the issues raised in Major Issue 1 above with regard to comparing to other methods, positioning, etc.**

   I have mentioned in the discussion that once the template is developed, it can simply be applied to all acquired data (lines 697-700):

   Although the image processing techniques described here can be difficult, once an analysis template has been developed in the MIFC software, batch processing allows for data files to be automatically analyzed, eliminating user intervention and therefore, scorer bias.

   I have added more information on the points you mention in the last paragraph of the introduction to give the readers an idea of wet lab time required and that the analysis template doesn't have to be re-created for each sample (lines 115-121):

   The sample processing technique used in this work requires less than 2 h to process a single sample and is relatively easy to perform in comparison to other methods. The data analysis in the MIFC analysis software is complicated, but creation of the analysis template can be accomplished in a few hours following the steps outlined in this paper. Moreover, once the template has been created, it can be automatically applied to all collected data without any further work.

9. **Pg 16, ln 700 - I believe you meant to say "Mitomycin C and Colchicine".**

   Yes I did, thanks for catching this. I've corrected it.

10. **Pg 17, ln 735 - Re-word the phrase/sentence "that MIFC method to perform".**

    I've re-worded this sentence so it now reads (line 740):

    This paper shows that the MIFC method developed to perform…

11. **Figure 5/Table 2 - Was there a specific rationale used to only go to a top concentration of MMC that elicited an approximate 30% cytotoxicity when the guidelines state that you can go as high at 55 +/- 5%? I recognize that this was observed to induce a statistically significant response, but the overall magnitude was relatively low - just under 3-fold compared to control - a level often invoked as a positive response. Also consider adding symbols to your tables to show which concentrations were determined to be statistically significantly different from control.**

    The Cytometry A paper (2018) shows that without the use of Cyt-B, a cytotoxicity of 61.8% was achieved at 0.5 µg/mL using Mitomycin C when using RPD as the metric to quantify toxicity. So, this dose range did actually exceed the 55±5% guideline. However, in the same paper, when examining the same dose range in the presence of Cyt-B using CBPI as the metric to quantify toxicity, a cytotoxicity of only 35% was observed. The latter data is the plot that appears in this manuscript.

    Other published studies have shown similar cytotoxicity results for several chemicals when quantifying MN increases in both the presence and absence of Cyt-B (e.g. Elhajouji, 2010, Mutation Research, 702: 157-162; Fellows *et al*., 2008, Mutation Research, 655: 4-21 and Fellows and Donovan, 2010, Mutation Research, 702: 163-170) presumably because the method to determine cytotoxicity is different. These results, along with the previously mentioned literature that warns of potentially false negative or positive results when not using Cyt-B, illustrate the importance of performing these types of experiments in both the presence and absence of Cyt-B.

    I have added symbols in the tables indicating the doses which produced statistically significant MN increases.

12. **Figure 5/Table 3 - Is there typically a variable baseline in the control MN frequency across studies? The mannitol study appears to be less than half what was observed for colchicine or MMC. How might this impact historical control ranges, criteria for establishing a positive response, etc?**

    The baseline frequency for the two negative control cultures in both the Mitomycin C and Colchicine experiments are 0.36%. For Mannitol the average MN frequencies across the entire dose range is 0.21%. So yes, the frequencies in the Mannitol study are about half of what was observed in the other two studies. Based on historically reported negative frequency ranges, I don't see this as a concern but I do see it as a reason why performing each experiment with at least two solvent control cultures is imperative.

    Lovell *et al*. recently published two studies examining historical control group data for TK6 cells (Mutat Res Gen Tox En, 2018, 825: 40-50) and human lymphocytes (Mutat Res Gen Tox En, 2019, 837: 52-59). For TK6 cells, results were examined from 13 laboratories and the range of MN frequencies in negative control samples varied between 0.32% and 1.38%. For human lymphocytes, 8 laboratories were examined and the range was even larger, between 0.22 and 1.59%.
    Therefore, the MN frequencies from solvent controls and all Mannitol cultures shown here are on the lower end of the ranges observed by Lovell *et al*., but the range is smaller and is much more consistent between experiments than the data they compiled from other laboratories using microscopy or flow cytometry.

Regarding how this impacts criteria for establishing a positive response, I think the results complied by Lovell *et al*. illustrates the importance of establishing a database of negative control MN frequencies within all laboratories. Lovell *et al*. recommend establishing these baseline frequencies from at least 20 experiments (presumably with and without solvent controls, and at various passages if using a cell line) to create a control database and determine the range of MN frequencies. I am currently working on creating this database for TK6 cells in our laboratory. The results are likely not sufficient to write a stand-alone publication on, but will be necessary to quantify positive responses in large scale studies that must be performed, which you point out in major concern #3.

Mask	Details
Binucleated	Levelset(M07, Ch07, Middle, 3)
	Dilate(Levelset(M07, Ch07, Middle, 3) 2)
	Watershed (Dilate(Levelset(M07, Ch07, Middle, 3) 2))
	Range(Watershed (Dilate(Levelset(M07, Ch07, Middle, 3) 2)),115-5000, 0.4-1)
	Component(1, Area, BN cell mask, Descending)
	Component(2, Area, BN cell mask, Descending)
Polynucleated	Levelset(M07, Ch07, Middle, 3)
	Dilate(Levelset(M07, Ch07, Middle, 3) 2)
	Watershed (Dilate(Levelset(M07, Ch07, Middle, 3) 2))
	Range(Watershed (Dilate(Levelset(M07, Ch07, Middle, 3) 2)),135-5000, 0.4-1)
	Component(1, Area, Poly cell mask, Descending)
	Component(2, Area, Poly cell mask, Descending)
	Component(3, Area, Poly cell mask, Descending)
	Component(4, Area, Poly cell mask, Descending)
Micronucleus	Spot(M07, Ch07, Bright, 2, 6, 2)
	Range(LevelSet(M07, Ch07, Middle, 3), 80-5000, 0-1)
	Dilate(Range(LevelSet(M07, Ch07, Middle, 3), 80-5000, 0-1), 2)
	Spot(M07, Ch07, Bright, 2, 6, 2) And Not Dilate(Range(LevelSet(M07, Ch07, Middle, 3), 80-5000, 0-1), 2)
	Spot Mask 1: Range(Spot(M07, Ch07, Bright, 2, 6, 2) And Not Dilate(Range(LevelSet(M07, Ch07, Middle, 3), 80-5000, 0-1), 2)), 10-80, 0.4-1)
	Spot(M07, Ch07, Bright, 2, 6, 1)
	Morphology(M07, Ch07)
	Dilate(Morphology(M07, Ch07), 3)
	Spot(M07, Ch07, Bright, 2, 6, 1) And Not Dilate(Morphology(M07, Ch07), 3)
	Spot Mask 2: Range(Spot(M07, Ch07, Bright, 2, 6, 1) And Not Dilate(Morphology(M07, Ch07), 3)) 8-80, 0.4-1)
	MN mask: Spot Mask 1 OR Spot Mask 2
Micronucleus component masks	Component(1, Area, MN mask, Descending)
	Component(2, Area, MN mask, Descending)
	Component(3, Area, MN mask, Descending)
	MN components mask: Component(1, Area, MN mask, Descending) Or Component(2, Area, MN mask, Descending) Or Component(3, Area, MN mask, Descending)
Additional micronucleus masks	Dilate(BNC mask, 4)
	Skeleton(Dilate(BNC mask, 4), Ch07, Thin)
	Dilate(Skeleton(Dilate(BNC mask, 4), Ch07, Thin), 1)
	Dilate(Skeleton(Dilate(BNC mask, 4), Ch07, Thin), 1) or MN components mask

	AdaptiveErode(M01, Ch01, 85)
	Dilate(AdaptiveErode(M01, Ch01, 85), 4)
	Dilate(AdaptiveErode(M01, Ch01, 85), 4) or MN components mask

PROTOCOL:

1. Preparation of Culture Medium and Culturing of TK6 cells

1.1. Prepare 565 mL of 1x RPMI culture medium. Add 5 mL of MEM non-essential amino acids (100x), 5 mL of sodium pyruvate (100 mM), 5 mL of penicillin-streptomycin-glutamine (100x), and 50 mL of fetal bovine serum (FBS) to a 500 mL bottle of RPMI 1640 1x medium. Prepare the medium in a biosafety cabinet and store at 2-8 °C. Heat the medium to 37 °C prior to adding it to the TK6 cells.

1.2. Thaw 1 mL of TK6 cells (stored at -80°C in DMSO) in 10 mL of medium. Centrifuge the cells at 200 x g for 8 min and aspirate the supernatant. Transfer the cells to 50 mL of media and incubate at 37 °C, 5% CO_2. The doubling time of TK6 cells varies from ~12-18 hrs and a few (3 or 4) passages will be required for the cells to reach their maximum proliferation rate (doubling time of about 14-15 hr).

1.3. Once the cells have reached their optimal proliferation rate, culture 100 mL of cells to a concentration of ~7-8 x 10^5 cells/mL.

2. Preparation of clastogens and/or aneugens and Cytochalasin B

2.1. Prepare appropriate stock concentrations of desired clastogens and aneugens. Stock concentrations must be sufficiently low such that chemicals do not precipitate. For example, for Mitomycin C, dissolve a full 2 mg bottle in 10 mL of sterile water to achieve a final stock concentration of 200 µg/mL. Mitomycin C can be stored at 4°C for three months.

2.2. On experiment day, prepare dilutions of the desired chemicals that are either 10-fold higher than the desired exposure concentrations if diluting in sterile water or 100-fold higher than the desired exposure concentrations if diluting in DMSO. For example, with Mitomycin C, prepare 3 mL dilutions in sterile water of 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 µg/mL.

2.3. Prepare a 200 µg/mL stock concentration of Cytochalasin B by dissolving a 5 mg bottle into 25 mL of DMSO. Cytochalasin B can be stored at -20°C for several months.

2.3.1. Note: Cytochalasin B is highly toxic. Inhaling, swallowing or having Cytochalasin B contact skin can be fatal. Wear appropriate personal protective equipment including a laboratory coat and two pairs of nitrile gloves. Wash hands thoroughly after handling.

3. Exposure of cells to clastogens and/or aneugens

3.1. Add 1 mL of desired clastogen or aneugen to 9 mL of cells at ~7-8x10^5 cells/mL in a T25 flask. For the control samples, add 1 mL of sterile water. Prepare at least two flasks per dose point to perform the experiment in duplicate. Place the flasks in a 37 °C, 5% CO_2 incubator for 3 hr.

3.1.1. **Note**: if chemicals are diluted in DMSO, add only 100 µL of the desired clastogen or

aneugen to each flask and add 100 µL of DMSO to controls. Each flask should contain 9.900 mL of cells.

3.2. After 3 hr, remove flasks from the incubator and transfer cells to 15 mL polypropylene tubes. Centrifuge at 200 x g for 8 min, aspirate the supernatant and transfer cells to new T25 flasks containing a total of 10 mL of fresh culture medium. Add 150 µL of the stock concentration (200 µg/mL) of Cytochalasin B to each flask to achieve a final concentration of 3 µg/mL.

3.3. Return the flasks to the 37 °C, 5% CO_2 incubator for a recovery time equal to 1.5-2.0 doubling times as recommended by the OECD guidelines[12].

3.3.1. **Note**: In this work the doubling time of the TK6 cells used was 15 hr, so a recovery time of 24 hr (1.6 doubling times) was used. Recovery times of less than 1.5 doubling times will not allow nuclear division to take place in samples exposed to higher doses impacting the number of scorable BN cells. Conversely, recovery times of more than 2.0 will produce a disproportionate number of polynucleated cells in samples exposed to lower doses, skewing the cytotoxicity calculations.

4. Preparation of buffers for fixation and labeling of DNA content

4.1. Prepare a 75 mM solution of potassium chloride (KCl) by adding 2.79 g to 500 mL of ultrapure water. Stir the solution for 5 min using a magnetic stirrer to thoroughly dissolve the KCl powder and sterile filter the solution through a 200 µm filter. The 75 mM KCl solution can be stored at 4°C for several months.

4.2. Prepare a sufficient amount of 4% formalin for the experiment, anticipating that a total of 2.1 mL must be added to each sample. For example, to prepare 10 mL of 4% formalin, add 4 mL of 10% formalin stock to 6 mL of 1x Dulbecco's Phosphate-Buffered Saline solution without Ca^{2+} or Mg^{2+} (PBS). This 4% formalin can be stored at room temperature for several weeks.

4.2.1. **Note**: CAUTION. Formalin/formaldehyde is toxic if inhaled or swallowed; is irritating to the eyes, respiratory system, and skin; and may cause sensitization by inhalation or skin contact. There is a risk of serious damage to eyes. It is a potential carcinogen.

4.3. Prepare 510 mL of wash buffer (2% FBS in 1X PBS) by adding 10 mL of FBS to a 500 mL bottle of 1x PBS.

4.4. Prepare 10 mL of a 100 µg/mL concentration of Hoechst 33342 by adding 100 µL of the stock concentration (1 mg/mL) to 9,900 µL of 1X PBS. The Hoechst 33342 solution can be stored at 4°C for several months.

5. Sample processing: hypotonic swelling, fixation, cell counting and labeling DNA content

5.1. At the end of the recovery period, remove all flasks from the incubator and transfer all samples to 15 mL polypropylene tubes. Centrifuge all samples at 200 x g for 8 min.

5.1.1. **Note**: depending on the clastogen or aneugen used, it may be necessary to perform a series of separate experiments to determine a dose range that is appropriate such that the top dose does not exceed a cytotoxicity of 55±5%[12].

5.2. Aspirate the supernatant, resuspend the cells and add 5 mL of 75 mM KCl. Mix gently by inversion three times and incubate at 4°C for 7 min.

5.3. Add 2 mL of 4% formalin to each sample, mix gently by inversion three times and incubate at 4°C for 10 min. This step acts as a "soft fixation".

5.4. Centrifuge all samples at 200 x g for 8 min. Aspirate the supernatant and resuspend in 100 µL of 4% formalin for 20 min. This step acts as a "hard fixation".

5.5. Add 5 mL of wash buffer and centrifuge at 200 x g for 8 min. Aspirate the supernatant and resuspend in 100 µL of wash buffer. Transfer all samples to 1.5 mL Eppendorf tubes.

5.6. Perform a cell count on each sample to determine the number of cells per sample. Samples will be highly concentrated so a 1:100 dilution in 1X PBS (10 µL of sample in 990 µL of PBS) will likely be required to obtain an accurate count.

5.6.1. **Note**: it is best to perform these cell counts using a hemocytometer. The addition of KCl gives the cytoplasm a translucent appearance making it difficult for automated cell counters to recognize them. Also, the use of Cyt-B increases the size of some cells, making it difficult for automated counters to accurately detect them.

5.7. If not running the samples on the MIFC immediately, they can be stored at 4°C for several days. When ready to run samples, add 5 µL of 100 µg/mL per $1x10^6$ cells/mL to each sample. Also add 10 µL of 500 µg/mL of RNase per 100 µL of sample for a final concentration of 50 µg/mL. Incubate the samples at 37°C, 5% CO_2 for 30 min.

5.8. Micro-centrifuge all samples at 200 x g for 8 min and carefully use a pipette to remove the supernatant such that approximately 30 µL of sample remains. Use a pipette to resuspend all samples before running on the MIFC ensuring there are no bubbles in the tube. **Do not vortex**. This ensures that all samples are highly concentrated and will minimize acquisition time.

6. Starting and calibrating the MIFC

6.1. Ensure the sheath, system calibration reagent, debubbler, cleanser and sterilizer containers (see the **Table of Materials**) are full and the waste tank is empty.

6.2. Power up the system and double-click on the MIFC software icon (see the **Table of Materials**).

6.3. Click the Startup button and make sure that the "Start all calibrations and tests" checkbox is checked. This will flush the system, load sheath and system calibration reagents, and calibrate the system.

7. Running samples on the MIFC

7.1. Launch the MIFC data acquisition software (see the **Table of Materials).**

7.2. Figure **1** shows the instrument settings. Turn on the 405 nm laser and set the laser power to 10 mW (**A**). Disable all other lasers (including SSC) and set the BF to channels 1 and 9 (**B**). Confirm that the magnification slider is set to 60X (**C**), high-sensitivity mode is selected (**D**), and that only channels 1, 7 and 9 are showing in the image gallery.

7.2.1. **Note**: If using a one camera MIFC, set the BF to channel 6 and ensure that only channels 1 and 6 are showing in the image gallery.

7.3. Click on the **Scatterplot** icon to create a scatterplot. Select the "All" population and select "Area M01" on the X-axis and "Aspect Ratio M01" on the Y-axis. Click on the **Square Region** icon and draw a region around the single cells. Name this region "Single Cells". Right click on the plot and select **Regions**. Highlight the "Single Cells" region and change the x-coordinates to 100 and 900 and change the y-coordinates to 0.75 and 1.

7.3.1. **Note**: If using a one camera MIFC, select "Area M06" on the X-axis and "Aspect Ratio M06" on the Y-axis.

7.4. Click on the **Scatterplot** icon to create a scatterplot. Select the "Single Cells" population and select "Gradient RMS M01" on the X-axis and "Gradient RMS M07" on the Y-axis. Click on the **Square Region** icon and draw a region around the majority of the cells. Name this region "Focused Cells". Right click on the plot and select **Regions**. Highlight the "Focused Cells" region and change the x-coordinates to 55 and 75 and change the y-coordinates to 9.5 and 15.

7.4.1. **Note**: If using a one camera MIFC with the BF in channel 6, select "Gradient RMS M06" on the X-axis and "Gradient RMS M01" on the Y-axis.

7.5. Click on the **Histogram** icon to create a histogram. Select the "Focused Cells" population and select "Intensity M07" as the feature. Click on the **Linear Region** icon and draw a region across the main peak in the histogram. Name this region "DNA-positive". Right click on the plot and select **Regions**. Highlight the "DNA-positive" region and change the coordinates to $2x10^5$ and $2x10^6$.

7.5.1. **Note 1**: The range of this region may have to be adjusted depending on the intensity. The idea is to eliminate very dimly stained cells at the low end of the intensity histogram.

7.5.2. **Note 2**: If using a one camera MIFC, then use the Intensity M01 feature.

7.6. Set the acquisition parameters (**Fig. 1E**). Specify the file name and the destination folder, change the number of events to 20,000 and select the "DNA-positive" population.

7.7. Click **Load** and place the control sample into the MIFC (**Fig. 1F**). Click the **Acquire** button to collect the data (**Fig. 1G**). Once the acquisition is complete, click the **Return** button to return the sample (**Fig. 1H**). Remove the sample tube from the instrument. Repeat this process for all remaining samples in the experiment.

8. Opening a data file in IDEAS

8.1. Launch the IDEAS software package (see the **Table of Materials**).

8.2. Click on Start Analysis to start the **Open File Wizard**. Select a data file by browsing to the desired raw image file (.rif). Click the **Open** button and click **Next**.

8.3. Since this is a single color assay, compensation is not necessary so click **Next** to bypass the compensation step. At this stage there is no analysis template to apply, so click **Next** again.

8.3.1. **Note**: If using the analysis template downloaded from the supplementary material, select it now. These templates only work with a 2 camera MIFC and the BF must have been set to channels 1 and 9 during acquisition.

8.4. Name the compensated image file (.cif) and the data analysis file (.daf). By default, the .cif and .daf file names are automatically generated to match the .rif. It is not recommended to change the names of the .cif and .daf. Click **Next**. Set the image display properties by selecting the "01" and "07" (or "01" and "06" if using a one camera MIFC). Click **Next**. There is no wizard that can be used for this application, so click **Finish**.

Note: sections 9 – 12 are very long and only selected key steps are shown in the main text of this paper. The full step-by-step analysis to create all masks, features, dot plots, histograms, custom image views and statistics reports can be found in Supplement 1 – Full Protocol.

Note: it is very important to save the data analysis file (.daf) and the analysis template (.ast) often during this protocol to avoid loss of progress.

9. Creating masks and features to identify BNCs

9.1. Start by clicking on the **Image Gallery Properties** icon (blue/white sun icon under the File

tab). Under the “Display Properties” click “Set Range to Pixel Data” then change the color to yellow. Click **OK**. This makes the Hoechst images easier to view since the purple color is difficult to see against the black background.

9.2. To identify non-apoptotic cells create an “Area-Threshold” feature plot it against the “BF contrast” feature:

9.2.1. Click on the **Analysis** tab, then click **Masks**. Click **New** then click **Function**. Under **Function** choose “Threshold”, under **Mask** choose “M07” and set the “Intensity Percentage” to 50. Click **OK** then **OK** again. Click **Close**.

9.2.2. Click on the **Analysis** tab, click **Features** then click **New**. For the **Feature Type** select “Area”. For **Mask** select the “Threshold(M07,Ch07,50)”. Click **Set Default Name** and click **OK**. Click on **Close** and IDEAS will start calculating the feature values.

9.2.3. Click on the **Dot Plot** icon. Select the “All” population. For the **X-axis feature** choose the “Contrast_M01_Ch01” feature and for the **Y-axis feature** choose the “Area_Threshold(M07,Ch07,50). Click **OK**.

9.2.4. Click the **Square Region** button and draw a region around the majority of the cells. Call this region “Non-apoptotic”. Right click on the plot and click **Regions**. Highlight the “Non-apoptotic” region. Set the **x-coordinates** to 0 and 15 and set the **y-coordinates** to 50 and 300. Click **Close**.

9.3. Create the BNC mask (steps 9.3.1 - 9.3.5) to identify cells that contain only two nuclei.

9.3.1. Browse for a BNC in the image gallery and click on it. This is to visualize creation of the mask in the Hoechst channel. Click on the **Analysis** tab, then click **Masks**. Click **New** then click **Function**.

9.3.2. Under **Function** choose “LevelSet”, under **Mask** choose “M07”, select the “Middle Level Mask” radio button and set the **Contour Detail Scale** to 3.00. Click **OK** then **OK** again. The mask is added to the list on the left.

9.3.3. Click **New** then click **Function**. Under **Function** choose “Dilate”, under **Mask** choose “LevelSet(M07,Ch07,Middle,3)”. Set the **image to display** to “Ch07”, and set the “Number of Pixels” to 2. Click **OK** then **OK** again.

9.3.4. Click **New** then click **Function**. Under **Function** choose “Watershed”, under **Mask** choose “Dilate(LevelSet(M07,Ch07,Middle,3)2)”. Set the **image to display** to “Ch07”, and set the “Line Thickness” to 1. Click **OK** then **OK** again.

9.3.5. Click **New** then click **Function**. Under **Function** choose “Range”, under **Mask** choose “Watershed(Dilate(LevelSet(M07,Ch07,Middle,3)2))”. Set the **image to display** to

"Ch07". Set the minimum and maximum area values to 115 and 5000 respectively. Set the minimum and maximum aspect ratio values to 0.4 and 1 respectively. Click **OK.** In the **Name** field change the text to read "BNC" then click **OK**. The BNC mask is complete.

9.4. Create the features and plots to obtain the final BNC population

9.4.1. Spot Count BNC feature: Click on the **Analysis** tab, then **Features**, then **New**. For the **Feature Type** select "Spot Count". For **Mask**, select the final BNC mask created in 9.3.5. Set the **Connectedness** to "Four" and change the name to "Spot Count BNC". Click **OK** then **Close** to calculate the feature values.

9.4.2. Spot Count BNC histogram. Click the **Histogram** icon. Select the "Non-apoptotic" population. For the **X-axis feature** choose the "Spot Count BNC" feature. Click OK. Click on the Linear Region icon. Draw a region across bin "2" on the histogram created in 9.4.2. Call this region "2N"

9.4.3. BNC Component Masks:

9.4.3.1. BNC Component Mask 1: Click on the **Analysis** tab, then **Masks**, then **New**, then **Function**. Under **Function** select "Component", under **Mask** select the "BNC" mask. For **Ranking Feature** select "Area", for **Sorting Order** click the "Descending" radio button. Set **Rank** to "1". Click **OK**.

9.4.3.2. BNC Component Mask 2: Repeat all steps in 9.4.3.1 except set Rank to "2". Click **Close**.

9.4.4. Aspect Ratio Intensity Features: Click on the **Analysis** tab, then **Features**, then **Add Multiple Features**. In the new window under **Select Base Features** check the "Aspect Ratio Intensity" box, under **Select Masks** choose both the "Component(1,Area,BNC,Descending)" and "Component(2,Area,BNC,Descending)" masks and under **Select Image** choose "Ch07". Click **Add Features**. In the new window click **OK**. Click **Close**, then **Close** again to start calculating the feature values.

9.4.5. *Circular Nuclei plot*. Click the **Dot Plot** icon. Select the "2N" population. For the **X-axis feature** choose the "Aspect Ratio Intensity_Component(1, Area, BNC, Descending)" feature. Change the **X-axis title** to "Aspect Ratio Intensity Component 1 (BNC)". For the **Y-axis feature** choose the "Aspect Ratio Intensity_Component(2, Area, BNC, Descending)" feature. Change the **Y-axis title** to "Aspect Ratio Intensity Component 2 (BNC)" Click **OK**. Click the **Square Region** button and draw a region around the majority of the cells. Call this region "Circular Nuclei". Right click on the plot and click **Regions**. Highlight the "Circular Nuclei" region. Set the **x-coordinates** to 0.75 and 1 and set the **y-coordinates** to 0.75 and 1. Click **Close**.

9.4.6. Component Area Ratio Feature: Click on the **Analysis** tab, then **Features**, then **Add Multiple Features**. In the new window under **Select Base Features** check the "Area" box, under **Select Masks** choose both the "Component(1,Area,BNC,Descending)" and "Component(2,Area,BNC,Descending)" masks and under **Select Image** choose "Ch07". Click **Add Features**. In the new window click **OK**. Click **Close**, then **Close** again to start calculating the feature values. When the calculations are complete, click on the **Analysis** tab, then **Features**, then **New**. Under **Feature Type** select the "Combined" radio button. In the **Features** box choose the "Area_Component(2, Area, BNC mask, Descending) feature and click on the down arrow to insert it. Click on the "/" button to insert the division operator. In the **Features** box choose the "Area_Component(1, Area, BNC mask, Descending)" feature and click on the down arrow to insert it. Under **Name**, type "Component Area Ratio (BNC)". Click **OK**. Click **Close**.

9.4.7. Component Intensity Feature: Click on the **Analysis** tab, then **Features**, then **Add Multiple Features**. In the new window under **Select Base Features** check both the "Spot Intensity Min" and "Spot Intensity Max" boxes, under **Select Masks** choose the "BNC" mask and under **Select Image** choose "Ch07". Click **Add Features**. In the new window click **OK**. Click **Close**, then **Close** again to start calculating the feature values. When the calculations are complete, click on the **Analysis** tab, then Features**,** then **New**. Under **Feature Type** select the "Combined" radio button. In the **Features** box choose the "Spot Intensity Min_Ch07" feature and click on the down arrow to insert it. Click on the "/" button to insert the division operator. In the **Features** box choose the "Spot Intensity Max_Ch07" feature and click on the down arrow to insert it. Under **Name**, type "Component Intensity Ratio (BNC)". Click **OK**. Click **Close**.

9.4.8. *Similar Area/Intensity plot*. Click the **Dot Plot** icon. Select the "Circular Nuclei" population. For the **X-axis feature** choose the "Component Intensity Ratio (BNC)" feature. For the **Y-axis feature** choose the "Component Area Ratio (BNC)" feature. Click **OK**. Click the **Square Region** button and draw a region around the majority of the cells. Call this region "Similar Area/Intensity". Right click on the plot and click **Regions**. Highlight the "Similar Area/Intensity" region. Set the **x-coordinates** to 0.75 and 1 and set the **y-coordinates** to 0.75 and 1. Click **Close**.

9.4.9. Aspect Ratio and Shape Ratio Features: Click on the **Analysis** tab, then **Features**, then **Add Multiple Features**. In the new window under **Select Base Features** check both the "Aspect Ratio" and "Shape Ratio" boxes, under **Select Masks** choose the "BNC" mask and under **Select Image** choose "Ch07". Click **Add Features**. In the new window click **OK**. Click **Close**, then **Close** again to start calculating the feature values.

9.4.10. *Separated Nuclei*. Click the **Dot Plot** icon. Select the "Similar Area/Intensity" population. For the **X-axis feature** choose the "Shape Ratio (BNC)" feature. For the **Y-axis feature** choose the "Aspect Ratio (BNC)" feature. Click **OK**. Click the **Square**

Region button and draw a region around the majority of the cells. Call this region "Separated Nuclei". Right click on the plot and click **Regions**. Highlight the "Separated Nuclei" region. Set the **x-coordinates** to 0.2 and 0.6 and set the **y-coordinates** to 0.3 and 0.55. Click **Close**.

9.4.11. Average BNC Circularity Feature: Click on the **Analysis** tab, then **Features**, then **Add Multiple Features**. In the new window under **Select Base Features** check the "Circularity" box, under **Select Masks** choose both the "Component(1,Area,BNC,Descending)" and "Component(2,Area,BNC,Descending)" masks and under **Select Image** choose "Ch07". Click **Add Features** and in the new window click **OK**. Click **Close**, then **Close** again to start calculating the feature values. Click on the **Analysis** tab, then **Features**, then **New**. Click the "Combined" radio button. Click the "(" button to insert an open parentheses. From the **Features** list, highlight "Circularity_Component(1, Area, BNC, Descending)" and click on the down arrow to insert it. Click the "+" symbol. From the **Features** list, highlight "Circularity_Component(2, Area, BNC, Descending)" and click on the down arrow to insert it. Click the ")" button to insert a closed parentheses. Click on the "/" button to insert the division operator. Beside the down arrow that does not have a drop down list, type the number "2". Press the down arrow beside the "2" to insert it into the formula. Under **Name**, type "Circularity BNC Avg". Click **OK**. Click **Close**.

9.4.12. Area BNC/Area M07 Ratio Feature: Click on the **Analysis** tab then **Features**, then **New**. Under **Feature Type** select "Area". Under **Mask** select "BNC". Click **Set Default Name** and click **OK**. Click **New** then click the "Combined" ratio button. Highlight the "Area_BNC" feature and click the down arrow to insert it. Click the "/" button to insert the division symbol. Highlight the "Area_M07" and click the down arrow to insert it. Click **Set Default Name** and click **OK**. Click **Close** again to start calculating the feature values.

9.4.13. BNCs plot. Click the **Dot Plot** icon. Select the "Separated Nuclei" population. For the **X-axis feature** choose the "Circularity BNC Avg" feature. For the **Y-axis feature** choose the "Area_BNC/Area_M07" feature. Click **OK**. Click the **Square Region** button and draw a region around the majority of the cells. Call this region "BNCs". Right click on the plot and click **Regions**. Highlight the "BNCs" region. Set the **x-coordinates** to 7 and 45 and set the **y-coordinates** to 0.6 and 1. Click **Close**.

10. Creating masks and features to identify MN within the BNC population

10.1. Create the MN mask (steps 10.1.1 – 10.1.4.1).

10.1.1. Browse for a BNC that contains an MN in the image gallery and click on it. This is to visualize creation of the MN mask in the Hoechst channel. Click on the **Analysis** tab, then click **Masks**.

10.1.2. Create Spot Identification Mask 1:

10.1.2.1. Click **New** then click **Function**. Under **Function** choose “Spot” and ensure the “Bright” radio button is selected. Under **Mask** choose “M07”, set the **Spot to Cell Background Ratio** to “2.00”. Set the **Minimum Radius** to “2” and the **Maximum Radius** to “6”. Click **OK** then **OK** again. The mask is added to the list on the left.

10.1.2.2. Click **New** then click **Function**. Under **Function** choose “Range”, under **Mask** choose “LevelSet(M07,Ch07,Middle,3)”. Set the **Image to display** to “Ch07”. Set the **Minimum Area** to 80 and the **Maximum Area** to 5000. Set the **Minimum Aspect Ratio** to 0 and the **Maximum Aspect Ratio** to 1. Click **OK** then **OK** again.

10.1.2.3. Click **New** then click **Function**. Under **Function** choose “Dilate”, under **Mask** choose “Range(LevelSet(M07,Ch07,Middle,3),80-5000,0-1)”. Set the **Image to display** to “Ch07”. Set the **Number of Pixels** to “2”. Click **OK** then **OK** again.

10.1.2.4. Click **New**. Double Click on the “Spot(M07, Ch07, Bright, 2, 6, 2)” mask to add it to the mask definition. Click the “And” Boolean operator button and then the “Not” Boolean operator button. Double click the “Dilate(Range(LevelSet(M07, Ch07, Middle, 3), 80-5000, 0-1), 2)” mask to add it to the mask definition. Click **OK**.

10.1.2.5. Click **New**, then **Function**. Under **Function** choose “Range” and under mask choose the mask created in 10.1.2.4:
“Spot(M07, Ch07, Bright, 2, 6, 2)” And Not “Dilate(Range(LevelSet(M07, Ch07, Middle, 3), 80-5000, 0-1), 2)”.
Set the **Image to Display** to “Ch07”. Set the **Minimum Area** to 10 and the **Maximum Area** to 80. Set the **Minimum Aspect Ratio** to 0.4 and the **Maximum Aspect Ratio** to 1. Click **OK** then **OK** again. *Spot identification mask 1 is complete*.

10.1.3. Create Spot Identification Mask 2:

10.1.3.1. Click **New** then click **Function**. Under **Function** choose “Spot” and ensure the “Bright” radio button is selected. Under **Mask** choose “M07”, set the **Spot to Cell Background Ratio** to “2.00”. Set the **Minimum Radius** to “1” and the **Maximum Radius** to “6”. Click **OK** then **OK** again.

10.1.3.2. Click **New** then click **Function**. Under **Function** choose “Morphology”, under **Mask** choose “M07”. Click **OK** then **OK** again.

10.1.3.3. Click **New** then click **Function**. Under **Function** choose “Dilate”, under **Mask**

choose “Morphology(M07, Ch07)”. Set the **Image to display** to “Ch07”. Set the **Number of Pixels** to “3”. Click **OK** then **OK** again.

10.1.3.4. Click **New**. Double Click on the “Spot(M07, Ch07, Bright, 2, 6, 1)” mask to add it to the mask definition. Click the “And” Boolean operator button and then the “Not” Boolean operator button. Double click the “Dilate(Morphology(M07, Ch07), 3)” mask to add it to the mask definition. Click **OK**.

10.1.3.5. Click **New**, then **Function**. Under **Function** choose “Range” and under mask choose the mask created in 10.1.3.4:
“Spot(M07, Ch07, Bright, 2, 6, 1)” And Not “Dilate(Morphology(M07, Ch07), 3”.
Set the **Image to Display** to “Ch07”. Set the **Minimum Area** to 8 and the **Maximum Area** to 80. Set the **Minimum Aspect Ratio** to 0.4 and the **Maximum Aspect Ratio** to 1. Click **OK** then **OK** again. *Spot identification mask 2 is complete*.

10.1.4. Create the final MN mask:

10.1.4.1. Click **New**. Double click on spot identification mask 1:
“Range(Spot(M07, Ch07, Bright, 2, 6, 2) And Not Dilate(Range(LevelSet(M07, Ch07, Middle, 3), 80-5000, 0-1), 2), 10-80, 0.4-1)” to add it to the mask definition.
Click the “OR” Boolean operator button. Double click on spot identification mask 2:
“Range(Spot(M07, Ch07, Bright, 2, 6, 1) And Not Dilate(Morphology(M07, Ch07), 3), 8-80, 0.4-1)” to add it to the mask definition. Change the **Name** to “MN Mask” and click **OK**. *The MN mask is complete*.

10.1.5. Create individual MN component masks:

10.1.5.1. MN Component mask 1: In the Mask Manager window click **New** then **Function**. Under Function select “Component”, under **Mask** select the “MN mask”. For **Ranking Feature** select “Area”, for **Sorting Order** click the “Descending” radio button. Set **Rank** to “1”. Click **OK**.

10.1.5.2. MN Component Masks 2-4: Repeat all steps in 10.1.5.1 except set Rank to “2” then “3” then “4”. This gives a total of four independent component MN masks:
“Component(1, Area, MN Mask, Descending)”
“Component(2, Area, MN Mask, Descending)”
“Component(3, Area, MN Mask, Descending)”

10.1.6. Create the MN Components Mask:

10.1.6.1. In the Mask Manager window click **New**. Double click on the "Component(1, Area, MN Mask, Descending)" mask to add it to the mask definition. Click on the "Or" Boolean operator button.
Double click on the "Component(2, Area, MN Mask, Descending)" mask to add it to the mask definition. Click on the "Or" Boolean operator button.
Double click on the "Component(3, Area, MN Mask, Descending)" mask to add it to the mask definition. Click on the "Or" Boolean operator button. Click **OK**. Click **Close**.

Note: it is extremely rare to encounter BNCs with more than 3 MN. However, additional component masks can be created by following the steps in 10.1.5.1 and 10.1.5.2. These masks can then be added to the "MN Components" mask by following 10.1.6.1.

10.1.7. Create the Skeleton mask to remove false positive artifacts between the nuclei:

10.1.7.1. Click on the **Analysis** tab then click **Masks**. Click **New** then click **Function**. Under **Function** choose the "Dilate" mask. Under **Mask** choose the "BNC m" mask. Set the **Number of Pixels** to "4" and click **OK** and **OK** again.

10.1.7.2. Click **New** then **Function**. Under **Function** choose the "Skeleton" mask. Uncheck the "Link inputs" checkbox. Make sure the "Thin" radio button is checked. Under **Mask** choose the "Dilate(BNC, 4)" mask. Under **Image to Display** choose "Ch07" and under **Channel** choose "Ch07". Click **OK** and **OK** again.

10.1.7.3. Click **New** then **Function**. Under **Function** choose the "Dilate" mask. Uncheck the "Link inputs" checkbox. Under **Mask** choose the "Skeleton(Dilate(BNC, 4), Ch07, Thin)" mask. Under **Image to Display** choose "Ch07" and under **Channel** choose "Ch07". Set the **Number of Pixels** to "1". Click **OK** and **OK** again.

10.1.7.4. Click **New**. Double click on the "Dilate(Skeleton(Dilate(BNC, 4), Ch07, Thin), 1)" mask to add it to the mask definition. Click on the "Or" Boolean operator button. Double click on the "MN Components" mask to add it to the mask definition. Click **OK**. Click **Close**.

10.1.8. Create the Cytoplasm mask to ensure MN being identified are inside the cell:

10.1.8.1. Click on the **Analysis** tab then click **Masks**. Click **New** then click **Function**. Under Function choose "Adaptive Erode". Set the **Adaptive Erode Coefficient** to "85". Click **OK** and **OK** again.

10.1.8.2. Click **New** then **Function**. Under **Function** choose the "Dilate" mask. Uncheck the "Link inputs" checkbox. Under **Mask** choose the "AdaptiveErode (M01,Ch01,85)" mask. Under **Image to Display** choose "Ch01" and under **Channel** choose "Ch01". Set the **Number of Pixels** to "4". Click **OK** and **OK** again.

10.1.8.3. Click **New**. Double click on the "Dilate(AdaptiveErode(M01,Ch01,85),4)" mask to add it to the mask definition. Click on the "Or" Boolean operator button. Double click on the "MN Components" mask to add it to the mask definition. Click **OK**. Click **Close**.

10.2. Create the features, dot plots and histograms to identify the BNCs containing MN

10.2.1. MN Spot Count feature. Click on **Analysis**, then **Features**, then **New**. Under **Feature Type** ensure the "Single" radio button is selected and choose "Spot Count" from the dropdown list. Under **Mask** choose "MN mask" and set the Connectedness to "Eight". Click **Set Default Name** then click **OK**, then **Close** to begin calculating the feature values.

10.2.2. MN Spot Count Histogram. Click the **Histogram** icon to create a histogram. Select the "BNCs" population as the parent population and select "Spot Count_MN Mask_8" as the feature. Click **OK** to add the histogram to the analysis area. Click on the **Linear Region** icon and draw a region from about 0.5 to 4.5. Name this region "all MN". Right click on the plot and select **Regions**. Highlight the "all MN" region and change the X coordinates to 0.5 and 4.5. Click **Close**.

10.2.3. Create Features and Dot Plot to ensure MN are inside the cytoplasm.

10.2.3.1. MN Inside Cytoplasm Feature. Click **Analysis**, then **Features**, then **New**. Ensure the "Single" radio button is selected and choose "Spot Distance Min" from the dropdown list. Under **Mask** choose "Dilate(AdaptiveErode(M01, Ch01, 85), 4) Or MN Components" and click **Set Default Name** and then click **OK**.

10.2.3.2. MN between nuclei Feature. In the still open Feature Manager window, click **New**. Ensure the "Single" radio button is selected and choose "Spot Distance Min" from the dropdown list. Under **Mask** choose "Dilate(Skeleton(Dilate(BNC, 4), Ch07, Thin, 1) Or MN Components" and click **Set Default Name** and then click **OK**. Click Close to calculate the features in steps 10.2.3.1 and 10.2.3.2.

10.2.3.3. Dot Plot. Click the **Dot Plot** icon. Select "all MN" as the parent population. For the **X Axis Feature** choose "Spot Distance Min_Dilate(Skeleton(Dilate(BNC,

4), Ch07, Thin, 1) Or MN Components" and for the **Y Axis Feature** choose "Spot Distance Min_Dilate(AdaptiveErode(M01, Ch01, 85), 4) Or MN Components". Click the **Square Region** button and draw a region around the majority of the cells. Name this region "MN Inside Cytoplasm". Right click on the plot and click **Regions**. Highlight the "MN Inside Cytoplasm" region. Change the **X Coordinates** to 0.1 and 20 and change the **Y Coordinates** to -0.5 and 0.5. Click **Close**.

10.2.4. Create Features and Dot Plot to identify the final MN population.

10.2.4.1. Aspect Ratio MN Feature. Click the **Analysis** tab then **Features** then **New**. Ensure the "single" radio button is selected and under **Feature Type** choose "Aspect Ratio". Under **Mask** select the "MN mask". Click **Set Default Name** and click **OK**.

10.2.4.2. Median Pixel BNC/Median Pixel MN combined feature.

10.2.4.2.1. With the Feature Manager window still open click **New** then select "Median Pixel" as the **Feature Type**. Under **Mask** select "BNC" and under Image select "Ch07". Click **Set Default Name** and click **OK**.

10.2.4.2.2. With the Feature Manager window still open click **New** then select "Median Pixel" as the **Feature Type**. Under **Mask** select "MN_Mask" and under Image select "Ch07". Click **Set Default Name** and click **OK**.

10.2.4.2.3. With the Feature Manager window still open click **New** then select the "combined" radio button under **Feature Type**. Select the "Median Pixel_BNC_Ch07" and click the down arrow to add it to the mask description. Click the division icon (/) to add the division symbol to the feature. Next, select the "Median Pixel_MN Mask_Ch07" and click the down arrow to add it to the mask description. Click **Set Default Name** and click **OK**. to calculate all features created in all steps in 10.2.4.

10.2.4.3. Dot Plot. Click the **Dot Plot** icon. Select "MN Inside Cytoplasm" as the parent population. For the **X Axis Feature** choose "Median Pixel_BNC_Ch07/Median Pixel_MN Mask_Ch07" and for the **Y Axis Feature** choose "Aspect Ratio_MN Mask". Click the **Square Region** button and draw a region around the majority of the cells. Name this region "MN BNCs". Right click on the plot and click **Regions**. Highlight the "MN BNCs" region. Change the **X Coordinates** to 1 and 5 and change the **Y Coordinates** to 0.6 and 1.0. Click **Close**.

11. Create masks, features and plots to identify the Mononucleated and Polynucleated populations

11.1. Create the POLY mask. Click **Analysis**, then **Masks**, then **New** then **Function**. Under **Function** choose “Range”, under **Mask** choose “Watershed(Dilate(LevelSet(M07, Ch07, Middle, 3), 2))”. Set the **image to display** to “Ch07”. Set the minimum and maximum area values to 135 and 5000 respectively. Set the minimum and maximum aspect ratio values to 0.4 and 1 respectively. Click **OK**. In the **Name** field change the text to read “POLY” then click **OK** then **Close**. The Polynucleated cell mask is complete.

11.2. Create the POLY Component Masks

11.2.1. POLY Component Mask 1: Click on the **Analysis** tab, then **Masks**, then **New**, then Function. Under **Function** select “Component”, under **Mask** select the “POLY” mask. For **Ranking Feature** select “Area”, for **Sorting Order** click the “Descending” radio button. Set **Rank** to “1”. Click **OK** then **OK** again.

11.2.2. POLY Component Mask 2: Repeat all steps in 11.2.1 except set Rank to “2”. Click Click **OK** then **OK** again.

11.2.3. POLY Component Mask 3: Repeat all steps in 11.2.1 except set Rank to “3”. Click Click **OK** then **OK** again.

11.2.4. POLY Component Mask 4: Repeat all steps in 11.2.1 except set Rank to “4”. Click Click **OK** then **OK** again. Click **Close** to close the mask manager.

11.3. Spot Count using POLY mask

11.3.1. Click the **Analysis** tab, then **Features**, then **New**. For **Feature Type**, select “Spot Count”. For **Mask** choose the “POLY” mask and set the Connectedness at “4”. Click **Set Default Name** and click **OK** then **Close** to calculate the feature values.

11.3.2. Click the Histogram icon. Select the “Non-apoptotic” population. For the **X-axis feature** choose the “Spot Count_POLY_4” feature.

11.3.3. MONO spot count region. Click on the Linear Region icon. Draw a region across bin “1” on the histogram created in 11.3.2. Call this region “1N”

11.3.4. TRI spot count region. Click on the Linear Region icon. Draw a region across bin “3” on the histogram created in 11.3.2. Call this region “3N”

11.3.5. QUAD MONO spot count region. Click on the Linear Region icon. Draw a region across bin “4” on the histogram created in 11.3.2. Call this region “4N”

11.4. Identify the MONO population

11.4.1. Create the MONO Aspect Ratio Feature. Click the **Analysis** tab, then **Features**, then

New. Under **Feature Type**, select the "Aspect Ratio" feature and under **Mask** select "Component(1, Area, POLY, Descending)". Click **Set Default Name** then click **OK**.

11.4.2. Create the MONO Circularity Feature. With the Feature Manager window still open, click **New**. Under **Feature Type**, select the "Circularity" feature and under **Mask** select "Component(1, Area, POLY, Descending)". Click **Set Default Name** then click **OK** then click **Close** to calculate the feature values.

11.4.3. Circular MONO cells dot plot. Click the **Dot Plot** icon. Select "1N" as the parent population. For the **X Axis Feature** choose "Circularity_Component(1, Area, POLY, Descending)" and for the **Y Axis Feature** choose "Aspect Ratio_Component(1, Area, POLY, Descending)". Click **OK**. Click the **Square Region** button and draw a region around the cell population towards the top right portion of the plot. Name this region "Circular_1N". Right click on the plot and click **Regions**. Highlight the "Circular_1N" region. Change the **X Coordinates** to 20 and 55 and change the **Y Coordinates** to 0.85 and 1.0. Click **Close**.

11.4.4. Create the Area POLY/Area_M07 feature. Click the **Analysis** tab, then **Features**, then **New**. Under **Feature Type**, select the "Area" feature and under **Mask** select "Component(1, Area, POLY, Descending)". Click **Set Default Name** then click **OK**. Click With the Feature Manager window still open, click **New** then under Feature Type click the "Combined" radio button. From the list of features, highlight the "Area_Component(1, Area, POLY, Descending)" and click the down arrow to add it to the feature definition. Click the division symbol (/). Select the Area_M07 feature and click the down arrow to add it to the feature definition. Click **Set Default Name** and click **OK**. Click **Close** to start calculating the feature values.

11.4.5. Final MONO population dot plot. Click the **dot plot** icon. Select "Circular_1N" as the parent population. For the **X Axis Feature** choose "Aspect Ratio_M07" and for the **Y Axis Feature** choose "Area_Component(1, Area, POLY, Descending) / Area_M07". Click **OK**. Click the **Square Region** button and draw a region around the majority of the cells. Name this region "Mononucleated". Right click on the plot and click **Regions**. Highlight the "Mononucleated" region. Change the **X Coordinates** to 0.85 and 1.0 and change the **Y Coordinates** to 0.55 and 1.0. Click **Close**.

11.5. Identify the trinucleated and quadranucleated cells

11.5.1. Create the Component Aspect Ratio and Circularity Features. Click the **Analysis** tab, then **Features**, then **Add Multiple Features**. Under **Select Base Features**, select the check boxes next to "Aspect Ratio" and "Circularity". Under Selected Masks select:
"Component(2, Area, POLY, Descending)"
"Component(3, Area, POLY, Descending)"
"Component(4, Area, POLY, Descending)"

Under Select Image choose “Ch07”. Click Add Features, then click OK. Verify that the following six features will be created:
“Aspect Ratio_Component(2, Area, POLY, Descending)”
“Aspect Ratio_Component(3, Area, POLY, Descending)”
“Aspect Ratio_Component(4, Area, POLY, Descending)”
“Circularity_Component(2, Area, POLY, Descending)”
“Circularity_Component(3, Area, POLY, Descending)”
“Circularity_Component(4, Area, POLY, Descending)”
Click **Close** to calculate the feature values.

11.5.2. Create the Average TRI Aspect Ratio Feature. Click the **Analysis** tab then **Features**, then **New**. Under **Feature Type**, click the “Combined” radio button. Click the open parenthesis button “(“ to add it to the feature definition.
Select the “Aspect Ratio_Component(1, Area, POLY, Descending)” feature and click the down arrow to add it to the feature definition. Click the plus (+) button to add it to the feature definition.
Select the “Aspect Ratio_Component(2, Area, POLY, Descending)” feature and click the down arrow to add it to the feature definition. Click the plus (+) button to add it to the feature definition.
Select the “Aspect Ratio_Component(3, Area, POLY, Descending)” feature and click the down arrow to add it to the feature definition. Click the close parenthesis “)” button to add it to the feature definition.
Click the division symbol “/” to add it to the feature definition. Type the number “3” into the box beside the arrow and click the arrow to insert it into the feature definition.
Under **Name** type “Aspect Ratio Trinucleated (Average)” and click **OK**.

11.5.3. Create the Average TRI Circularity Feature. Click the **Analysis** tab then **Features**, then **New**. Under **Feature Type**, click the “Combined” radio button. Click the open parenthesis button “(“ to add it to the feature definition.
Select the “Circularity_Component(1, Area, POLY, Descending)” feature and click the down arrow to add it to the feature definition. Click the plus (+) button to add it to the feature definition.
Select the “Circularity_Component(2, Area, POLY, Descending)” feature and click the down arrow to add it to the feature definition. Click the plus (+) button to add it to the feature definition.
Select the “Circularity_Component(3, Area, POLY, Descending)” feature and click the down arrow to add it to the feature definition. Click the close parenthesis “)” button to add it to the feature definition.
Click the division symbol “/” to add it to the feature definition. Type the number “3” into the box beside the arrow and click the arrow to insert it into the feature definition.
Under Name type “Circularity Trinucleated (Average)” and click **OK** then click **Close**.

11.5.4. Trinucleated Cell dot plot. Click the **dot plot** icon. Select “3N” as the parent population. For the **X Axis Feature** choose “Circularity Trinucleated (Average)” and for the **Y Axis Feature** choose “Aspect Ratio Trinucleated (Average)”. Click **OK**. Click the **Square Region** button and draw a region around cells near the top right of the plot. Name this region “Trinucleated”. Right click on the plot and click **Regions**. Highlight the “Trinucleated” region. Change the **X Coordinates** to 13 and 35 and change the **Y Coordinates** to 0.85 and 1.0. Click **Close**.

11.5.5. Create the Average QUAD Aspect Ratio Feature. Click the **Analysis** tab then **Features**, then **New**. Under **Feature Type**, click the “Combined” radio button. Click the open parenthesis button “(“ to add it to the feature definition.
Select the “Aspect Ratio_Component(1, Area, POLY, Descending)” feature and click the down arrow to add it to the feature definition. Click the plus (+) button to add it to the feature definition.
Select the “Aspect Ratio_Component(2, Area, POLY, Descending)” feature and click the down arrow to add it to the feature definition. Click the plus (+) button to add it to the feature definition.
Select the “Aspect Ratio_Component(3, Area, POLY, Descending)” feature and click the down arrow to add it to the feature definition. Click the plus (+) button to add it to the feature definition.
Select the “Aspect Ratio_Component(4, Area, POLY, Descending)” feature and click the down arrow to add it to the feature definition. Click the close parenthesis “)” button to add it to the feature definition.
Click the division symbol “/” to add it to the feature definition. Type the number “4” into the box beside the arrow and click the arrow to insert it into the feature definition.
Under **Name** type “Aspect Ratio Quadranucleated (Average)” and click **OK**.

11.5.6. Create the Average QUAD Circularity Feature. Click the **Analysis** tab then **Features**, then **New**. Under **Feature Type**, click the “Combined” radio button. Click the open parenthesis button “(“ to add it to the feature definition.
Select the “Circularity_Component(1, Area, POLY, Descending)” feature and click the down arrow to add it to the feature definition. Click the plus (+) button to add it to the feature definition.
Select the “Circularity_Component(2, Area, POLY, Descending)” feature and click the down arrow to add it to the feature definition. Click the plus (+) button to add it to the feature definition.
Select the “Circularity_Component(3, Area, POLY, Descending)” feature and click the down arrow to add it to the feature definition. Click the plus (+) button to add it to the feature definition.
Select the “Circularity_Component(4, Area, POLY, Descending)” feature and click the down arrow to add it to the feature definition. Click the close parenthesis “)” button to add it to the feature definition.
Click the division symbol “/” to add it to the feature definition. Type the number “4”

into the box beside the arrow and click the arrow to insert it into the feature definition. Under **Name** type “Circularity Quadranucleated (Average)” and click **OK** then click **Close**.

11.5.7. Quadranucleated Cell dot plot. Click the **dot plot** icon. Select “4N” as the parent population. For the **X Axis Feature** choose “Circularity Quadranucleated (Average)” and for the **Y Axis Feature** choose “Aspect Ratio Quadranucleated (Average)”. Click **OK**. Click the **Square Region** button and draw a region around cells near the top right of the plot. Name this region “Quadranucleated”. Right click on the plot and click **Regions**. Highlight the “Trinucleated” region. Change the **X Coordinates** to 13 and 35 and change the **Y Coordinates** to 0.85 and 1.0. Click **Close**.

12. Create a custom view to examine the BNC and MN masks

12.1. Click on the **Image Gallery Properties** button (Blue and White icon) then click on the **View** tab. Click on the **Composites** tab then click **New**. Under **Name** type “Ch01/Ch07”. Click **Add** Image. Under **Image** choose “Ch01” and set the **Percent** to 100. Click **Add Image** again, under **Image** choose “Ch07” and set the **Percent** to 100.

12.2. Click **New** and under Name type “BNC and MN masks”

12.3. Click **Add Column**. Under **Image Type** choose “Ch01” and under **Mask** choose “None”

12.4. Click **Add Column**. Under **Image Type** choose “Ch07” and under **Mask** choose “None”

12.5. Click **Add Column**. Under **Image Type** choose “Ch07” and under **Mask** choose “BNC”

12.6. Click **Add Column**. Under **Image Type** choose “Ch07” and under **Mask** choose “MN mask”

12.7. Click **Add Column**. Under **Image Type** click the “Composite” radio button. The “Ch01/Ch07” composite image should be automatically added to the view. Click **OK** to close the Image Gallery Properties window.

12.8. To display the custom view, click the **View** pulldown menu and select “BNC and MN masks”. Click the **Show/Hide Masks** button to display the BNC and MN masks on top of the Ch07 images.

13. Create a custom view to examine the POLY mask

13.1. Click on the **Image Gallery Properties** button (Blue and White icon) then click on the **View** tab.

13.2. Click **New** and under Name type “POLY mask”

13.3. Click **Add Column**. Under **Image Type** choose “Ch01” and under **Mask** choose “None”

13.4. Click **Add Column**. Under **Image Type** choose “Ch07” and under **Mask** choose “None”

13.5. Click **Add Column**. Under **Image Type** choose “Ch07” and under **Mask** choose “POLY”

13.6. Click **Add Column**. Under **Image Type** click the “Composite” radio button. The “Ch01/Ch07” composite image should be automatically added to the view. Click **OK** to close the Image Gallery Properties window.

13.7. To display the custom view, click the **View** pulldown menu and select “POLY mask”. Click the **Show/Hide Masks** button to display the BNC and MN masks on top of the Ch07 images.

14. Create a statistics table to enumerate key events

14.1. Click on the **Reports** tab then click “Define Statistics Report”. In the new window, click Add **Columns**.

14.2. Add the BNC count statistic. Under **Statistics** select “Count” and under **Selected Population** choose the “BNCs” population. Click “Add Statistics” to add the statistic to the list.

14.3. Add the MN count statistic. Under **Statistics** select “Count” and under **Selected Population** choose the “MN BNCs” population. Click “Add Statistics” to add the statistic to the list.

14.4. Add the MONO count statistic. Under **Statistics** select “Count” and under **Selected Population** choose the “Mononucleated” population. Click “Add Statistics” to add the statistic to the list.

14.5. Add the TRI count statistic. Under **Statistics** select “Count” and under **Selected Population** choose the “Trinucleated” population. Click “Add Statistics” to add the statistic to the list.

14.6. Add the QUAD count statistic. Under **Statistics** select “Count” and under **Selected** Population choose the “Quadranucleated” population. Click “Add Statistics” to add the statistic to the list. Click **Close** then click **OK**.

14.7. **Note**: to change how the columns are labeled in the statistics table, highlight each row in the table and uncheck the “Use default title” option. Then in the Column title field, type the desired column title.

14.8. Ensure that the statistics table is providing the correct values. Click the **Reports** tab then click “View Statistics Report”.

14.9. The data analysis template is complete. Save your template (File, Save as Template).

15. Batch process experiment files using the data analysis template

15.1. Open a new instance of the IDEAS software package. Under the **Tools** menu click "Batch Data Files" then click "Add Batch" in the new window.

15.2. In the new window click "Add Files" to select the experiment files (.rif) to add to the batch. Under the **Select a template or data analysis file (.ast, .daf)** option, click the open folder icon to browse to and open the data analysis template (.ast file) that was saved in step 14.9.

15.3. Click the **Preview Statistics Report** button to ensure the proper analysis template has been selected

15.3.1. **Note**: No values will be displayed here since they have not yet been calculated. However, this step serves as a check to ensure the proper analysis template has been selected prior to running the batch.

15.4. Click **OK** to close the current window. Then click **Submit Batches** to start the batch processing of all files.

15.5. When the batch processing has completed a .txt file will be available in the folder that contains all of the .rif files. The statistics can then be used to calculate genotoxicity and cytotoxicity.

16. Calculating the genotoxicity and cytotoxicity parameters

16.1. Calculating genotoxicity

16.1.1. To calculate genotoxicity, use the statistics table created in 15.5. Divide the number of cells in the "MN BNCs" population by the number of cells in the "BNC" population then multiply by 100:

$$\frac{MN\ BNCs}{BNCs} \times 100$$

16.2. Calculating cytotoxicity

16.2.1. Determine the total number of POLY cells by summing the number of TRI and QUAD cells.

16.2.2. Calculate the Cytokinesis-Block Proliferation Index (CBPI) by using the number of cells in the MONO, BNCs and POLY as follows:

$$CBPI = \frac{[(MONO) + (2 \times BNCs) + (3 \times POLY)]}{MONO + BNCs + POLY}$$

16.2.3. Finally, calculate the cytotoxicity of each culture by using the CBPI values from the control cultures (C) and chemically exposed culture (T) as follows:

$$Cytotoxicity = 100 - 100\left[\frac{(CBPI_T - 1)}{(CBPI_C - 1)}\right]$$

Section 7:
It is best to just show the creation of the plot in step 7.3 then say something along the lines of: "create the two remaining plots by following steps 7.4 and 7.5 in the protocol". I don't think it is necessary to show the creation of three plots in the video.

Section 9:
It might be best to show steps 9.3.1 and 9.3.2 then say something like "follow all steps in Section 9 in *Supplement 1 – Full Protocol* to create the masks, features and plots to identify the final BNC population. It is important to realize that region boundaries may have to be modified depending on several factors such as stain intensity and cell type used."

Section 10:
The creation of the micronucleus mask is very complicated. It may be best to show steps 10.1.2.1 and 10.2.1.4 in the video and then make a statement such as "to create the full MN mask, individual MN components masks and other MN-related masks please see steps 10.1.3 to 10.1.8.3 in *Supplement 1 – Full Protocol*."

Identifying the final micronucleus population also has some complicated steps so it might be good to show the creation of the spot count feature (step 10.2.1) and the spot count histogram (step 10.2.2) but then make a statement like "Follow steps 10.2.3 and 10.2.4.3 to create the remaining features and plots to identify the final micronucleated binucleated cell population".

Section 11:
There are many steps in the creation of the mononucleated and polynucleated masks so it would be good to show how the polynucleated mask is created (step 11.1). Following this a statement could be made along these lines: "To create the component masks, features and plots to identify mononucleated, trinucleated and quadranucleated cells, follow all steps in section 11 in *Supplement 1 – Full Protocol*."

Sections 12 and 13:
It would be good to show the first few steps in section 12 (12.1 – 12.4) then say something like "follow all the steps in sections 12 and 13 in *Supplement 1 – Full Protocol* to create custom views to show all masks" and then show what the custom views actually look like.

Section 14:
Here it should be sufficient to show how to add one statistic to the table and then say something like "follow the remaining steps in section 14 in *Supplement 1 – Full Protocol* to create the full statistics table". We can then show the final statistics table in the video.

Section 15:
Here we can show the first couple of steps to start the batch processing procedure then refer the viewer to all of the steps in section 15 in Supplement 1 – Full Protocol so they can complete the batch processing step.