A high-throughput *in vivo* micronucleus assay for genome instability screening in mice

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Abstract

We describe a sensitive, robust, high-throughput method for quantifying the formation of micronuclei, markers of genome instability, in mouse erythrocytes. Micronuclei are whole chromosomes or chromosome segments that have been separated from the nucleus. Other methods of detection rely on labour-intensive, microscopy-based techniques. Here, we describe a 2 day, 96-well plate based flow-cytometric method of micronucleus scoring that is simple enough for a research technician experienced in flow cytometry to perform. The assay detects low levels of genome instability that cannot be readily identified by classic phenotyping, using 25 µl of blood. Using this assay, we have screened >10,000 blood samples and discovered novel genes that contribute to vertebrate genome maintenance, as well as novel disease models and mechanisms. We discuss experimental design considerations, including statistical power calculation, provide troubleshooting tips, and discuss factors that contribute to a false-positive increase in numbers of micronucleated red blood cells and experimental variability.

Editorial summary: This protocol describes a sensitive, robust, high-throughput method for quantifying the formation of micronuclei in mouse erythrocytes. It detects low levels of genome instability and can be used to identify novel genome maintenance genes and models.

INTRODUCTION

Micronuclei are extra-nuclear segments of chromatin that can arise as a result of DNA double-strand breaks or mitotic-spindle-apparatus dysfunction. Micronuclei form infrequently during mitosis of healthy cells, and may either be acentric chromosomal fragments or whole chromosomes that have failed to be incorporated into the nucleus at the completion of mitosis[1] (Fig. 1a and 1b). The use of micronuclei as a means to quantify cytogenetic damage was originally proposed by Evans *et al*.[2]. Cells derived from patients with hereditary genome-instability disorders, such as Ataxia telangiectasia and Fanconi anemia, have a high frequency of micronucleus formation, and micronucleus assays can be used to aid the diagnosis of these disorders [3,4]. In addition, many genome instability disorders predispose to cancer, and genome instability is a hallmark of spontaneously arising cancers[5].

Recent genomic analyses have identified a novel form of genome instability in cancer cells, a phenomenon termed 'chromothripsis'[6]. Chromothripsis involves up to thousands of clustered chromosomal rearrangements, thought to occur as a single event and confined to genomic regions in one or a few chromosomes. This phenomenon has sparked further interest in micronuclei, which have been shown to undergo highly error-prone DNA replication and DNA breakage[7,8]. It is thought that DNA fragments could be reassembled in

the wrong order by the process of non-homologous end-joining, leading to massive chromosomal rearrangements in mitosis. Micronuclei have been shown to re-incorporate into the same nuclear compartment as the main chromosomes during mitosis[7]. This raises the possibility that genomic reintegration of a genomically rearranged micronucleus might occur in cancers and could be selected for if they cause an oncogenic event such as loss or disruption of a tumour suppressor gene[7,9,10].

Elevated levels of micronuclei have been associated with tumor predisposition in genetically modified mice[11-13], and micronucleus assays have been used to assess the carcinogenic potential of compounds in toxicity studies, and for biomonitoring mice and humans that have been exposed to mutagens such as radiation[14-17].

Development and applications of the method

Fliedner *et al.* first proposed the idea of scoring micronuclei as a biological dosimeter of radiation exposure in humans[18]. Measuring numbers of micronuclei in mammalian red blood cells is relatively straightforward because normoblasts (erythrocyte precursor cells that reside in the bone marrow) expel their nucleus during red blood cell development, leaving behind any micronuclei that have formed (Fig. 1a and 1b). In the blood, micronucleated erythrocytes were first identified by microscopy using a basophilic stain, and were called 'Howell-Jolly bodies' after the two scientists who first discovered them[19-22]. While micronucleated red blood cells are extremely rare in humans, in part due to their efficient clearance by the spleen, mice are less efficient at

clearing micronucleated red blood cells from the peripheral circulation, and so they are more readily detected in this model organism[23].

Hutter and Stohr[24] were the first to describe a flow cytometry–based method of scoring micronucleated erythrocytes from bone-marrow, which they compared with standard microscopy-based scoring. Their method involved the fixation of samples followed by the addition of the fluorescent DNA stain 4',6-diamidino-2-phenylindole (DAPI) and the protein fluorochrome Sulforhodamine 101 to discriminate between normal anuclear erythrocytes and micronucleated erythrocytes. Their method was later refined and applied to the analysis of peripheral erythrocytes using a variety of DNA stains, including DAPI, Hoechst and propidium iodide (PI)[25,26].

In mice, 1-3% –of red blood cells are reticulocytes (RET), immature erythrocytes that are characterized by their cytoplasmic reticular mesh of RNA and their large size, whereas the remaining 93-94% are mature normochromatic erythrocytes (NCE), which are characterized by their 'donut' shape and the absence of a nucleus (Fig. 1a). –In 1992, Grawé and co-workers described a method that distinguished between micronucleated RET, which are thiazole orange positive, and NCE using a dual-laser flow cytometer configuration[25,27]. The distinction between RET and NCE is important for assessing the effects of genotoxic challenge because micronucleated RET are indicative of recent damage, whereas micronucleated NCE are indicative of damage caused >72 hours earlier. RET can be distinguished from NCE by studying the expression of the mouse transferrin receptor (CD71), which is present on the surface of most dividing hematopoietic progenitor cells[28]. In

1996, Dertinger and co-workers refined the method using a fluorescein-conjugated monoclonal antibody to the CD71 receptor, which labels the RET population[29]. Using PI to stain DNA (after removal of RNA from RET by incubation with ribonuclease) simplified the assay by enabling the use of a single-laser flow cytometer for enumeration of cells (Patent US6100038A and US5858667A)[30].

In 2003, Shima *et al.* used the assay developed by Dertinger and co-workers to genetically screen mice for novel genes required for vertebrate genome maintenance[31,32]. Here, we have scaled-down Dertinger's method to perform the assay in 96-well plates (Fig. 2), which we have used to assess genome stability in >10,000 mice as part of a large-scale~~,~~ reverse genetic screen~~,~~ in association with the Mouse Genetics Project at the Wellcome Trust Sanger Institute, UK[33]. The program screens genetically modified mice as part of an international effort to associate gene function with developmental abnormalities and disease. By combining the assay with high-throughput phenotyping of mice, we have identified several novel mouse models of rare genome-instability disorders, novel genome maintenance genes, including *Cenpj* and *Mysm1*, and cancer predisposition genes including *Slx4*[11,34-38]. If such a screen were to be performed for the human population, albeit with a slightly modified method that enriches for RET prior to assay processing due to the efficient clearance of micronucleated erythrocytes by the human spleen[39], the assay could aid the diagnosis of novel genome instability syndromes or identify individuals that may be predisposed to cancer due to elevated basal levels of genome instability. Furthermore, by incorporating a

challenge with ionizing radiation into the mouse phenotyping pipeline, we have identified radiation-sensitive mutants, which exhibit increased levels of micronucleated-RET after irradiation but not before. Within this manuscript, we provide additional details on data analysis, troubleshooting and potential sources of false-positive increases in numbers of micronucleated red blood cells and data variability.

Our high-throughput flow-cytometric micronucleus assay is relatively quick, inexpensive, and simple to perform. It uses equipment that is available in most research organizations, and is reliable if performed according to the instructions provided herein. It therefore makes an ideal test for the detection of genome instability *in vivo* that is minimally invasive and does not require the death of the animal, meaning that it can easily be incorporated into a phenotyping screen. We provide step by-step technical advice, as well as a troubleshooting guide with tips gleaned from our use of the method over the past few years.

Limitations of the method

The high-throughput micronucleus assay gives an indication of genome stability as measured by the frequency of micronucleated erythrocytes in a population. The assay does not provide information on the structure of the micronucleus and therefore does not distinguish between the different mechanisms of action of inducers, such as clastogens or aneugens. Neither does the assay inform on whether a cell has a single micronucleus or multiple micronuclei, as could be determined by conventional microscopy. Therefore

further experiments are needed to determine the structure of the micronucleus and mechanism of its formation. For example, we have previously shown that fibroblasts derived from *Cenpj*-deficient mice (1.5-fold increase in % micronucleated normochormatic erythrocytes) are prone to tetraploidy, as shown by multiplex metaphase fluorescent *in situ* hybridization and indicated by cell cycle analysis[35]. This suggested that micronuclei were likely the result of chromosome missegregation; fluorescence microscopy revealed that fibroblasts had an increased frequency of supernumerary centrosomes and multi-polar spindles which have been shown to cause aneuploidy[35].

Alternatively, micronuclei can also be detected *in vitro* using the cytokinesis-block micronucleus cytome assay[40]. In addition to quantifying micronuclei, the cytokinesis-block micronucleus cytome assay also detects cytostasis, cytotoxicity and provides information on the structure of the micronucleus. This added information is useful for determining the likely mechanism of micronucleus formation. For example, nucleoplasmic bridges are a biomarker of misrepair and/or telomere end-fusions, whereas nuclear buds are a biomarker of elimination of amplified DNA and/or DNA repair complexes[40]. However, the cytokinesis-block micronucleus cytome assay is time-consuming and relies on a complicated scoring system. In summary, the high-throughput micronucleus assay is a fast and sensitive method for the detection of micronuclei, but further experiments are needed to ascertain the mechanism of its formation.

Through screening for micronucleus induction in parallel with a large-scale, unbiased mouse phenotyping pipeline, we have identified several factors that

may influence the detection of true, micronucleated red blood cells with the assay. Regenerative anemia causes increased production of red blood cells (reticulocytosis) and activation of extramedullary hematopoiesis[41]. In extreme cases of reticulocytosis, such as that observed in 16-20 week old C57BL/6J *Apc*$^{min/+}$ mice (Suppl. Fig. 1a-b), the main nucleus may not be expelled during erythropoiesis, resulting in erythroblastosis, which is the presence of nucleated red blood cells in the peripheral circulation. The assay does not distinguish between micronucleated and nucleated red blood cells and so this causes a false-positive increase in % micronucleated-NCE (Suppl. Fig. 1a-b). With the high-throughput micronucleus assay, there is a positive correlation between % RET and % micronucleated-NCE (Suppl. Fig. 1b). The use of an anti-CD71 transferrin receptor antibody enables the researcher to quantify reticulocytes to ensure that numbers are within normal limits (e.g. for healthy C57BL/6 mice, RET should constitute 1-2% of the total red blood cell population; Suppl. Fig. 1c). Furthermore, the assay relies on the distinction of blood cells by size and granularity, and therefore any changes to these parameters could result in a false-positive increase in the number of micronucleated-red blood cells. As a rule of thumb, if other changes in other hematopoietic parameters are observed, such as but not limited to red blood cell distribution width (or 'RDW'), we would confirm the micronucleated cell type using microscopy. A simple method to confirm a change in micronucleated-NCE would be to perform cell counts on fresh blood smears stained with May-Grumwald Giemsa (Fig. 1b) or on methanol-fixed blood samples co-stained with DAPI on slides (Fig. 1c and 1d). For the quantification of micronuclei from slides stained with May-Grunwald Giemsa,

we recommend counting >6000 red blood cells or cells from fifteen fields of view (40X magnification) per sample, for at least three mice per test group. For a wild-type mouse, we would expect to see 1-2 micronucleated cells/1000 erythrocytes. For a positive-control mouse, we would expect to see at least 2-4 micronucleated erythrocytes/1000 erythrocytes**.**

Experimental design

Test and control mice should be matched by sex, age and genetic background, since all of these factors influence micronucleus induction in mice (Box 1 and Fig. 3a-b). The assay is highly sensitive and reproducible if screening is performed with mice of the same sex that are bred on a pure genetic background (Fig. 3c). It is possible to detect minimal changes with statistical significance using the appropriate sample number (Box 1 and Fig. 3d). The simplest way to generate positive control animals for the assay is to expose wild-type mice to genotoxic agents, including treatment with 1 mg/kg mitomycin C or 0.75 Gray irradiation (Box 2; Fig. 4a-b, respectively). Alternatively, several mouse models with elevated micronucleus levels, such as the *Slx4*-null mouse (Fig. 4c-e), have previously been characterized and mice, or their blood samples, could be requested from the corresponding author of the study (see review by Balmus & McIntyre[42]). Importantly, the experimenter should ensure that the experiment is performed in accordance with local animal research regulations and ARRIVE guidelines[43].

MATERIALS

REAGENTS

- Laboratory mice (e.g. C57BL/6J mice from The Jackson Laboratory, (Maine, USA), #000664)
- Methanol (CH_3OH; e.g. Sigma-Aldrich, #494437)
- Heparin (e.g. Sigma-Aldrich, #H3393; ≥180 U.S.P. (U.S. Pharmacopeia) units/mg)
- Sodium chloride (NaCl; e.g. Sigma-Aldrich, #S3014)
- Sodium phosphate dibasic (HNa_2O_4P; e.g. Sigma-Aldrich, #S5136)
- Sodium bicarbonate ($CHNaO_3$; e.g. Sigma-Aldrich, #S6297)
- Hydrochloric acid (HCl; e.g. Sigma-Aldrich, #84415)
- Ribonuclease A from bovine pancreas (RNase A; e.g. Sigma-Aldrich, #R4642; activity ≥ 70 Kunitz units/mg protein)
- Fluorescein-isothiocyanate (FITC)-conjugated rat anti-mouse CD71 transferrin receptor antibody (e.g. Southern Biotech, #1720-02; 0.5 mg/ml)
- Mitomycin C from *Streptomyces caespitosus* (MMC; e.g. Sigma-Aldrich, #P4864) CRITICAL Only required if using MMC to produce positive control.
- Propidium iodide 1.0 mg/ml solution (PI; e.g. Sigma-Aldrich, # P4864)

EQUIPMENT

- Flow cytometer capable of handling multi-titer-well plates (e.g. 96-well plate) and equipped with 488 nm blue and 561 nm green lasers (e.g. LSR Fortessa™ with high-throughput sampler option, Becton Dickinson) or, alternatively, with only a 488 nm laser.

- Centrifuge with 4˚C setting (e.g. Eppendorf 5810R) or centrifuge located in a cold room
- -80˚C freezer
- pH meter (e.g. Meter Toledo MP220)
- Biological safety cabinet (e.g. Fisher Scientific, #12372419)
- Reciprocating shaker (e.g. Stuart SSL2)
- Aluminum foil (e.g. Terinex)
- Single-use 0.22 µm filters (e.g. Corning, #430521)
- 10 ml serological pipets (e.g. Corning - Product #4101)
- 15 ml polypropylene conical centrifuge tubes (e.g. Corning, #352097)
- 5 ml round bottom polystyrene tubes for flow cytometry (e.g. Corning, #352054)
- 96-deep-well plates with 0.8 ml capacity (e.g. ABgene, #AB-0859)
- 96-well clear flat bottom plate for flow cytometry assay (e.g. Corning, #353075)
- 1.5 ml microcentrifuge tubes (e.g. Eppendorf, #0030120191)
- Flow cytometry data analysis software (e.g. FlowJo, Tree Star, Inc.) and statistical analysis software (e.g. Prism, GraphPad)
- Optional: live animal irradiator (e.g. CIS-IBL 437 C; C^{137})
- Optional: sterile lithium-heparin-coated vacutainer blood-collection tubes (e.g. Microtainer, Beckton Dickinson, #365953)

REAGENT SETUP

Mitomycin C (MMC). Prepare MMC solution in a sterile laminar flow cabinet (Class II). Prepare 0.5 mg/ml stock solution with sterile water. Store at 4˚C for

up to two weeks. CAUTION: MMC is potentially mutagenic or carcinogenic; wear gloves, safety glasses and a laboratory coat when handling.

Heparin. Prepare a 500 U.S.P. units/ml (2.35 mg/ml Sigma-Aldrich H3393) solution in sterile PBS. Filter (0.22 µm filter) and aliquot 300 µl into 1.5 ml tubes and store at -20˚C for up to one year. Freeze-thaw no more than three times. The solution may be stored at 4˚C for several weeks in 1.5 ml tubes but evaporation occurs over longer periods.

Fixative. >12 hours before blood collection, aliquot 2 ml methanol into 15 ml conical tubes in a chemical safety cabinet and cap tightly. It is recommended to prepare at least two tubes per blood sample from each mouse, but to process one sample and keep the second sample for technical repetition or for back-up. Place tubes in -80˚C freezer for >12 hours and less than one month. We do not recommend long-term storage of methanol aliquots in the freezer, rather, methanol should be stored in a suitable 'flammables' cupboard away from sources of ignition. CAUTION: methanol is flammable, corrosive and toxic, so aliquot in a chemical safety hood away from sources of ignition, and wear gloves, safety glasses and a laboratory coat when handling.

Bicarbonate buffer. Prepare 0.9% NaCl, 5.3 mM sodium bicarbonate (84 g/mole), and bring to pH 7.5 by adding HCl. Autoclave and store at 4˚C for up to one year. A 10X stock solution can be prepared, autoclaved and stored at 4˚C for up to a year, but pH must be determined for the 1X solution again when diluted for use. CAUTION: HCl is corrosive so wear gloves, safety glasses and a laboratory coat when handling, and pipette reagent in a chemical safety cabinet. CRITICAL: Bicarbonate buffer must be cold before

use, so allow plenty of time for cooling. If fixed blood samples are washed with warm buffer, they will turn brown and will be unsuitable for analysis.

CD71/RNase solution. This solution should be freshly prepared. Working in dimmed lighting, prepare enough bicarbonate buffer/RNase/CD71-FITC antibody mix for the required number of samples plus an extra 10% ('dead' volume). To each sample, add 0.2 µl anti-CD71-FITC (0.5 mg/ml) and 7 µl RNase A (e.g. Sigma, #R4642, activity ≥ 70 Kunitz units/mg protein) to bicarbonate buffer (e.g. 72.8 µl) to make a total volume of 80 µl. To test the activity of new batches of RNase, we recommend performing a dilution series and comparing profiles after incubation with, for example, 0, 4, 8, 12 µl RNase. CRITICAL: Sufficient RNase A activity is required to digest the RNA present in reticulocytes; low-quality RNase A may lead to inaccurate results since PI binds to RNA as well as DNA (see Suppl. Fig. 2a-b for profiles associated with incomplete digestion of RNA). TROUBLESHOOTING

Animals. We obtain our mice from The Jackson Laboratory (Maine, USA). The care and use of all mice used to generate data for this protocol was carried out in accordance with UK Home Office regulations, UK Animals (Scientific Procedures) Act of 2013. Our mice are maintained in a specific pathogen free unit on a 12 hours light: 12 hours dark cycle with lights off at 7:30pm and no twilight period. The ambient temperature is 21±2˚C and the humidity is 55±10%. Mice are housed using a stocking density of 3–5 mice per cage (overall dimensions of caging: (LxWxH) 365x207x140 mm, floor area 530 cm^2) in individually ventilated caging (Tecniplast Seal Safe1284L) receiving 60 air changes per hour. In addition to Aspen bedding substrate, standard environmental enrichment of two nestlets, a cardboard Fun Tunnel

and three wooden chew blocks are provided. Mice are given water and diet ad libitum.

EQUIPMENT SETUP

Flow cytometer. Warm up the lasers for half an hour and prepare the instrument for high-throughput sample analysis using a 96-well plate (refer to the manufacturer's instrument setup guide). The fluorescence emitted from FITC-conjugated anti-CD71 and PI is collected through a 530/30 nm and a 610/20 nm band-pass filter after excitation by the blue 488 nm and the green 561 nm laser, respectively. Data resolution is improved and compensation is not required when the fluorochromes are excited and detected independently of each other using a 488 nm and 561 nm laser configuration. When a single 488 nm blue laser is used in this experiment, compensation is required during data acquisition since FITC and PI have a slight spectral overlap (please refer to reagent supplier technical sheet), the same excitation wavelength and the same detection light path (please refer to your instrument technical specification).

Set the high-throughput sampler to mix each sample two-to-four times before acquiring 200,000 events per sample at a rate of 1000-2000 events per second. The preparation of single-fluorochrome and unstained controls from surplus sample to setup the regional gates for the analysis of the erythrocyte cell population is included in the main protocol. CRITICAL STEP: maintain an acquisition rate of less than 2500 events per second on a volumetric flow rate of 0.5 µl-1 µl per second. A higher volumetric flow rate can result in an

increased rate of false positive events for the CD71-FITC-positive, PI-positive population. See Suppl. Fig. 2a and 2c. TROUBLESHOOTING.

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PROCEDURE

Blood collection, fixation and storage TIMING: approximately 10 minutes per mouse.

1 | Collect 50 µl of blood (for example, ~2-3 drops from a tail vein or retro-orbital bleed) into 300 µl heparin solution in a 1.5 ml tube[44]. Alternatively, blood can be collected into a heparinized vacutainer; however, it must then be transferred to the heparin solution to ensure proper single-cell dissociation of the cells prior to fixation. CAUTION: blood must be obtained from mice using a technique that is approved by local regulatory bodies and/or ethics committees[44].

CRITICAL: Ensure the blood sample is fixed within 12 hours of collection to minimize the risk of sample degradation, which is one of the most common sources of false positives (Suppl. Fig. 3). TROUBLESHOOTING.

2 | Rapidly expel 150 µl of heparin-blood mix directly over the 2 ml aliquot of cold methanol (taken straight out of -80°C freezer) using a pipette and cap the 15 ml tube. Repeat for duplicate tube. CRITICAL STEP: Do not put the pipette tip in the methanol and do not let the blood touch the side of the tube, or aggregation may occur. CRITICAL STEP: The temperature of the methanol must be -80˚C before fixation as warmer methanol causes the blood to turn red-brown (Suppl Fig. 2d) and alters the size and granularity of the blood cells, leading to a false positive increase in micronucleated-

erythrocytes (Suppl. Fig. 3). The blood sample should remain bright red in fixative. TROUBLESHOOTING.

3 | Invert the sample 4-5 times to prevent aggregation, and store the tubes at -80°C for at least 12 hours before continuing. We recommend that the tubes are returned to their original polystyrene packaging, as this provides added protection should the freezer fail. We do not recommend transferring samples to a -20°C freezer after fixation, as warmer methanol temperatures change the size and granularity of blood cells, which can result in a slight elevation of the micronucleated-NCE population (Suppl. Fig. 3). PAUSE POINT: When methanol fixed samples are stored at -80˚C, they remain stable for up to a year (Suppl. Fig. 2e and Suppl. Fig. 4a). At this stage, the 15 ml tubes of methanol fixed samples can be placed in a sealed bag, shipped on dry-ice and stored in a -80˚C freezer upon arrival at new destination.

Sample preparation TIMING 3-4 hours for 96 samples

4 | Working on ice, add 12 ml of cold (4˚C) bicarbonate buffer, cap and invert 15 ml tubes to mix. Centrifuge at 500 *g* for 5 minutes at 4 ˚C. CRITICAL STEP: samples must remain cold, especially while the cells are in undiluted methanol; if samples become warm at this stage they may turn red-brown (Suppl. Fig. 2f) the size and granularity of blood cells can change and the data may be uninterpretable (Suppl. Fig. 3). TROUBLE SHOOTING.

5 | Pour off the supernatant, add 200 µl cold bicarbonate buffer and resuspend the cell pellet by pipetting gently up and down.

6 | Transfer 20 µl of sample to each well in a 96 deep-well plate (800 µl/well capacity). Add 20 µl of any sample to three 1.5 ml microcentrifuge tubes for preparation of unstained control, PI-only control and CD71-FITC-only control, which are required for flow cytometry setup.

7 | Add 80 µl of CD71-FITC/RNase solution to test samples in the plate and to the CD71-FITC-single-stain control tube. Add 7 µl RNase and 73 µl buffer to the unstained control and PI-only control tubes.

TROUBLESHOOTING

8 | Cover plate with foil and incubate for 45-60 minutes at 4˚C with gentle agitation (approximately 60 rpm) on a shaker.

9 | Wash samples (in wells and eppendorf tubes) by addition of 600 µl cold bicarbonate buffer (4˚C) and centrifuge at 500 g for 5 minutes at 4˚C.

10 | Pour off supernatant by quickly inverting the plate over a waste container. The cells will remain in the wells. Pipette off supernatant from control tubes. Resuspend pellets in 500 µl bicarbonate buffer by pipetting gently up and down.

11 | Transfer 200 µl sample to a clear, flat-bottomed 96-well plate. –The remaining sample can be stored at 4˚C and used for technical replication if required.

Flow cytometric analysis TIMING: 2-3 hours for 96 samples

12 | Transfer 500 µl of each control sample into 5 ml round-bottom polystyrene tubes.

13 | Using the unstained control, acquire events with logarithmic side-scatter (SSC-A-log) *versus* linear forward scatter (FSC-A-Lin) axes. The SSC *versus* FSC plot should reveal two to three overlapping populations of red blood cells with reasonably well-defined edges. –Centre the main population (erythrocytes) using the voltage and gain settings, and create the first gate (R1) just inside the erythrocyte population (Fig. 5a).

14 | Isolate doublets by plotting FSC-W-Lin *versus* FSC-A-Lin and set gate R2 around single cells (Fig. 5b).

15 | Add 2.5 µl PI to the PI-only control tube, leave a few minutes and acquire events. Adjust the voltage and gain to ensure all events are within the axes and draw a quadrant gate to separate the positive and negative populations. Repeat for the CD71-FITC-only control.

16 | Use the PI single-stain control to identify events caused by auto-fluorescence (Suppl. Fig. 5c-d) and remove by setting gate R3 on the PI biexponential scale *versus* SSC biexponential scale axes (Fig. 5c and Ssuppl. Fig. 5d);

17 | On CD71-FITC-biexponetial *versus* PI-biexponential axes, gate the four different populations (RET – Q1; MN-RET- Q2; MN-NCE-Q3; NCE – Q4; Fig. 5d).

18 | Save the experiment settings on the flow cytometer.

19 | Switch the flow cytometer from tube mode to multi-titer-well plate mode.

20 | Set the volume to 70 µl, mixes to 4, volumetric flow rate to 0.5 µl or 1 µl per second (to achieve around 1000-2000 events/sec).

21 | Add 1 µl PI to each well of the plate using a multipipettor, loading 24 wells at a time. Loading more than 24 wells at a time may increase variability (see Suppl. Fig. 4b). Although pipetting 1 µl is not as accurate as pipetting larger volumes, our data suggest high assay reproducibility (see Box 1 for examples). We routinely use an 8-channel Biopette™ (1-10 µl) or an Eppendorf Multipette® plus. Mix by pipetting up-and-down with a multi-channel pipette (20-200 *u*l), incubate for a few minutes.

TROUBLESHOOTING

22 | Load the saved protocol, acquire and save data.

TROUBLESHOOTING

23 | Export data, repeat the analysis (create gate R1 around erythrocytes, isolate doublets and set gate R2, remove auto-fluorescence by setting gate R3, gate the four populations) using appropriate software (e.g. FlowJo, TreeStar Inc.).

24 | To calculate the % micronucleus (MN) formation use the absolute event counts for each of the indicated quadrants:

$$\% \text{ MN-RET} = [\text{MN-RET}/(\text{RET} + \text{MN-RET})] \times 100$$

$$\% \text{ MN-NCE} = [\text{MN-NCE}/(\text{NCE} + \text{MN-NCE})] \times 100$$

TIMING for 96 samples

Day 1 (reagent setup): Prepare bicarbonate buffer (10 minutes), autoclave and place at 4˚C. Label 192 15 ml tubes (96 x 2), aliquot 2 ml methanol into each and place at -80˚C (approximately 2 hours).

Day 2 (steps 1-3): Collect 96 blood samples into heparin solution. If experienced in the tail vein bleed technique, this should take approximately 4 hours. –Fix blood in methanol (-80˚C; approximately 1.5 hours).

Day 3 (steps 4-24): Wash samples in buffer, centrifuge and resuspend in buffer (approximately 2.5 hours), incubate for 45-60 minutes in CD71-FITC/RNase solution, wash in buffer and centrifuge (15 minutes), add PI and analyze by flow cytometry (approximately 2 hours).

TROUBLESHOOTING

See Table 1 for troubleshooting guidance.

ANTICIPATED RESULTS

The anticipated results with the flow cytometric micronucleus assay depend on the mouse genetic background, sex, age and level of exposure to genotoxic agents (Box 1; Fig. 3 and Fig. 4). Any significant increase over the wild type mouse baseline can be identified with high sensitivity using simple statistics that are appropriate for the experimental design (Box 1). The successful procedure is dependent on proficient sample collection, storage, preparation and acquisition on the flow-cytometer (see Troubleshooting). Upon sample collection the blood should not be allowed to coagulate and the fixation should be done in timely manner in -80°C methanol. Upon fixation the solution should have a distinctive bright red color that will preserve throughout storage; brown coloration will indicate defective fixation. During data acquisition on the flow cytometer the quality of the blood preparation can be easily observed on the graph generated on side scatter logarithmic scale

(SSC-Log) *versus* forward scatter linear scale (FSC-Lin) where the total blood has a distinctive pattern that is easily distinguishable (Fig. 5a); deviations from this pattern might indicate sample degradation or defective fixation (see Troubleshooting). The comparison between the unstained, CD71-only, PI-only and the CD71/PI stained samples will demonstrate successful sample staining. The addition of wild-type and positive controls to each plate (see Experimental design considerations) will confirm that the assay has been run successfully.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Author contributions

D.J.A. and S.P.J conceived the idea of screening mice from large-scale mouse phenotyping pipelines for micronucleated erythrocytes. G.B. and R.E.M. performed the experimental analysis and wrote the manuscript with comments from all authors. B.L.N. assisted with flow-cytometry and N.K. performed statistical analysis.

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Box 1 – Sources of variation in micronucleus frequency

Below we highlight significant sources of variation in micronucleus frequency that should be taken into consideration during experimental design.

Sex

For all mouse strains that we have tested, the frequency of micronucleated-NCE for males was significantly increased when compared to females. For example, female C57BL/6N mice have a mean of 0.14% micronucleated-NCE, whereas the mean for males is 0.21% (*P*=0.0002, mixed model analysis where sex is a fixed effect and assay date a random effect; Fig. 3a).

Genetic background

The assay is sensitive enough to detect significant differences between inbred strains of mice (Fig. 3b). Compared with 129SvEv mice (n = 27), C57BL/6JIco mice (n = 15) have a higher basal level of %micronucleated-NCE (0.186% compared with 0.143%; *P*=0.0065; mixed model analysis for which core strain is a fixed effect and assay date a random effect). Relative to the variance of

129SvEv (i.e. variance of 1), the variance of C57BL/6JIco is similar (relative variance=1.18), whereas the variance of mixed strains is more than three times greater (relative variance of B6Brd;129S5-Tyr$^{c\text{-}Brd}$ = 3.29, CBACa;129S5 = 3.63 ; Fig. 3b), suggesting that data variability is greater for mice with mixed genetic background than for those with pure genetic backgrounds. This finding should be considered when determining statistical power. It should be noted that, when comparing mixed genetic backgrounds comprised of sub-strains of laboratory mice, the variance is low (Fig. 3b). For example, if we compare the variance of a mixed strain of mice, where all strains are historically derived from BL/6 mice, then the variance is similar to that of a pure inbred strain (variance of B6Brd;B6Dnk;B6N-Tyr$^{c\text{-}Brd}$ =0.92 relative to the pure, inbred strain 129SvEv). In summary, assay sensitivity is highest when mice are of the same sex and are bred on a pure genetic background (Fig. 3c).

Age

DNA damage is thought to accumulate with age and, therefore, older mice may have higher frequencies of spontaneous micronucleus formation than young mice[45]. We recommend that mice are age-matched where possible.

Assay date

By performing the micronucleus assay as part of a large-scale phenotyping pipeline we have found that, as with other phenotyping variables, assay date is a significant source of variation[46]. The variation with date arises from multiple factors including researcher, reagent lot, cage, mother and litter size.

This temporal variation has a significant impact on data analysis as readings taken on the same day are more similar than readings on different days. The simplest approach to manage this source of variation is to process all samples at the same time, which will enable the use of standard statistical methods such as the Student's *t*-test. Where it is not possible to collect samples and process them on the same day, such as with a large-scale phenotyping pipeline, micronucleus data can be analyzed with a mixed model approach, where genotype is treated as a fixed effect and assay date as a random effect. This separates the genotype effect from the temporal variation by assuming that temporal variation is normally distributed with mean of zero and with a defined variance. We refer the reader to Karp *et al.* 2012 for further statistical design considerations with regard to assay date[46].

Statistical power

Data from 200 blood samples obtained from male C57BL/6N wild-type mice, which were screened over seven assay dates, were used to determine the appropriate statistical method and associated statistical power. Across the seven datasets, the % micronucleated-NCE data was normally distributed, as assessed by graphical inspection (normal Q-Q plot) and Shapiro-Wilk normality test (data not shown). These data characteristics indicate that a standard Student's *t*-test is appropriate to detect changes in the mean % micronucleated-NCE between the control group and test group, subject to the experimental design implemented. The average standard deviation was estimated to be 0.026%.

Using the variance estimate, we assessed the power to detect changes of ±0.05%, 0.1% and 0.2% micronucleated-NCE for different samples sizes when using a two-tailed, two-sampled Student's *t*-test[47]. For hypothesis testing of age-, sex- and genetic-background-matched test and control mice, we recommend a target power of >0.8. For confirmation of genomic instability mutants, we suggest a power ≥ 0.95 (Fig. 3d). The power calculations demonstrated that the assay is very sensitive and that n = 3 mice per genotype is enough to test for differences >0.1% micronucleated-NCE (approximately 1.5-fold change for C57BL/6N mice) in a confirmation experiment, and that n = 6 mice per genotype are required to test differences as low as 0.05% micronucleated-NCE (approximately 1.25-fold change for C57BL/6N mice) in a screening experiment. The variance of mixed genetic backgrounds is greater than for pure genetic backgrounds and this will reduce the statistical power (Fig. 3b).

Box 2 – DNA damage response challenge: generation of positive control samples and detection of molecules involved in the response to exogenous DNA damaging agents

DNA damage response (DDR) defects can be revealed by treating mice that show normal spontaneous micronucleus frequencies with genotoxic agents. Upon treatment, micronuclei form in mitotically dividing erythroid progenitor cells and can be detected 24-72 hours later in peripheral reticulocytes (Fig. 1 and Fig. 4a and 4b). This procedure can be used to create positive control samples from wild-type control animals or for the identification of deficiencies that are only apparent, or amplified, in response to DDR challenge because they are only necessary for the response to exogenous DNA damaging agents rather than endogenous DNA damaging agents. This procedure will enable the researcher to detect genes that are not necessary for the response to endogenous DDR stress, thus their deregulation does not produce elevated spontaneous micronucleus levels, but are critical for the response to DNA damage.

PROCEDURE TIMING: 3 days.

1 | Before genotoxic challenge, collect and fix 50 µl of blood as detailed in the spontaneous micronucleus procedure (steps 1-3); include an untreated control cohort to ensure that the procedure does not cause marked reticulocytosis (see 'limitations of the method' and TROUBLESHOOTING).

2 | Challenge the animals with the specific genotoxic agent; the recommended dose of irradiation is 0.75 Gy whole-body irradiation and the

recommended dose of mitomycin C is 1 mg/kg body weight *via* intraperitoneal injection.

3 | Collect and fix 50 µl of blood 24, 48 and 72 hours post-treatment as detailed in the spontaneous micronucleus procedure (steps 1-3).

4 | Continue as detailed in the spontaneous micronucleus procedure (steps 4-24);

ANTICIPATED RESULTS: Wild-type mice treated with these genotoxins show a significant increase in the %micronucleated-RET 24 and 48 hours post-treatment and recovery is observed 72 hours post treatment (Fig. 4a and 4b). For example, 48 hours after 0.75 Gy, wild-type mice have a mean 5-fold increase in % micronucleated-RET (baseline mean = 0.61% for n = 10; 48 hours after 0.75 Gy mean = 3.05% for n = 3), whereas 48 hours after administration of 1 mg/kg mitomycin C, wild-type mice have a 7-fold increase (baseline mean = 0.61% for n = 10; 48 hours after treatment mean = 4.23% for n = 3). Genotoxin sensitivity is characterized by a normal % micronucleated-RET frequency before treatment but a significant increase in % micronucleated-RET *versus* control, 48 hours post-treatment. Fig. 4b demonstrates the radiosensitivity of *Akt2*$^{-/-}$ (*tm1a[EUCOMM]WTSI*) mice. Treatment must be carried out in accordance with the local regulations for the use of animals in research.

Figure legends

Figure 1. Micronucleus induction during erythropoiesis. a) Erythropoiesis is characterized by maturation of the multipotent hematopoietic stem cell (not shown) to a smaller, mitotically inactive normoblast. Within the normoblast, chromatin begins to condense, and eventually the pyknotic nucleus is expelled, yielding a cell that is known as a reticulocyte. The reticuloctyes (RET) migrate from the bone marrow to the peripheral circulation, where, one or two days later, it matures into a normochromatic erythrocyte (NCE), which is characterized by degradation of the reticular RNA mesh and loss of transferrin receptor (CD71) expression. Micronuclei can form spontaneously during the mitotic stages of erythropoiesis, for example, within the multipotent hematopoietic stem cell or differentiated precursor cells (normoblasts); however, in normal cells this is an extremely rare event. While the nucleus is expelled during red blood cell maturation, the micronucleus is retained. If cells are genomically unstable or mice have been treated with a genotoxin, the frequency of micronucleus formation increases. Micronuclei are propidium iodide (PI) positive and can be differentially identified in NCE or RET by co-staining with CD71. b) May-Grumwald Giemsa staining of fresh blood sample from a wild-type mouse shows a Giemsa positive DNA fragment (micronucleus) in an erythrocyte. c + d) DAPI stained micronucleated-erythrocyte from a blood sample prepared according to the methods indicated in this protocol (heparin anticoagulation followed by fixation in -80˚C methanol). The methanol-fixed blood was cyto-spun onto a slide and DAPI stained (panel c~~|~~ shows staining with overlay phase-contrast/DAPI, whereas panel d~~|~~ shows staining with DAPI alone). Size bars 5 µm. Arrows indicate micronuclei.

Figure 2. Workflow of micronucleus assay. Mouse blood samples are collected into liquid heparin solution and fixed in cold methanol. Samples are washed in bicarbonate buffer, cells are pelleted and resuspended in buffer, before incubation in bicarbonate buffer containing FITC-conjugated anti-transferrin receptor (CD71) antibody and RNase. Samples are washed out of the incubation solution with bicarbonate buffer, and resuspended in buffer before adding propidium iodide (PI) and collecting 200 000 events by flow cytometry.

Figure 32. Effect of sex and genetic background on micronucleus induction and determination of statistical power. a) Wild-type female mice have significantly decreased %micronucleated normochromatic erythrocytes (%MN-NCE; mean ± standard deviation: 0.141 ± 0.031; n = 20) relative to wild-type males (mean ± standard deviation: 0.209 ± 0.03; n = 20; *P* = 0.0002, mixed model analysis with assay date as a random effect and sex as a fixed effect). b) Graph shows %MN-NCE for 16 week old male mice bred on different genetic backgrounds; the assay is sensitive enough to detect significant differences between strains. Data represents mean (bar) and standard deviation (error bar): 0.144 ± 0.024, n = 26 129SvEv *versus* 0.186 ± 0.025 n = 14 BL6/JIco, *P*=0.002, mixed model analysis with assay date as a random effect and core strain as a fixed effect; 0.208 ± 0.023, n = 46 B6Brd;B6Dnk;B6N-Tyr$^{c\text{-}Brd}$; 0.160 ± 0.051, n=10 B6Brd;129S5-Tyr$^{c\text{-}Brd}$; 0.188 ± 0.077, n = 10 CBACa;129P2. Note the increased variance for mixed genetic backgrounds e.g. 129P2xCBACa, compared to mixed genetic substrains e.g.

B6Brd;B6Dnk;B6N-Tyr$^{c\text{-}Brd}$. c) Graph shows data for five technical replicates for blood samples obtained from three C57BL/6 wild type male mice. Data demonstrate that there is good reproducibility when using mice of the same sex and genetic background. Data shows mean (bar) and error bars represent standard deviation. d) Power analysis shows the estimated power as the number of biological replicates increases. The relationship was studied for three effect sizes changes (absolute difference of 0.05, 0.1 or 0.2% MN-NCE). The dotted lines highlight the target power of 0.8 for a hypothesis generating experiment or 0.95 for a hypothesis testing experiment.

Figure 43. Micronucleus induction after genotoxic treatment or genetic modification. a) Graph shows mean and standard deviation for induction of micronucleus (MN) formation in reticulocytes (RET) of wild-type mice treated with the DNA crosslinking agent mitomycin C (MMC; 1 mg/kg; n=3) or 0.75 Gy ionizing radiation (IR; n = 4), relative to untreated mice (n = 4). Mice were bled at the indicated time points, and % MN-RET was calculated as a percentage of total RET. Forty eight hours after MMC or IR treatment, %MN-RET is significantly increased relative to untreated control mice (*P<0.01, Student's two-tailed t-test[AU: State what error bars are.]). b) Graph shows mean and standard deviation for n≥5 *Akt*$^{+/+}$; *Akt*$^{+/-}$ and *Akt*$^{-/-}$ male mice after 0.75 Gy IR. *Akt*$^{-/-}$ mice have a higher frequency of %MN-RET after IR, when compared to littermate controls (*P<0.0001; two-tailed Student's t-test). c-e) Increased %MN-normochromatic erythrocytes (NCE) in a mouse model of Fanconi anaemia**.** Blood was collected from *Slx4*$^{+/+}$ (c) and *Slx4*$^{-/-}$ (d) mice at 16 weeks of age and processed with the high-throughput micronucleus assay protocol.

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Representative plots are shown. Note the increased frequency of MN-NCE in blood obtained from *Slx4*$^{-/-}$ mice (d) when compared to that of control *Slx4*$^{+/+}$ mice (c). e) Graph shows MN-NCE as a percentage of total NCE. *Slx4*$^{-/-}$ mice have significantly higher levels of spontaneous micronucleus induction than *Slx4*$^{+/+}$ mice (mean ± standard deviation: 0.21 ± 0.025; Slx4$^{+/+}$ *versus* 0.51 ± 0.078; Slx4$^{-/-}$; n = 6, B6Brd;B6Dnk;B6N-Tyr$^{c\text{-}Brd}$ males for each genotype; *P<0.0001, Student's two-tailed *t*-test). Data is duplicated from Crossan *et al.* 2011[11] to provide an example of a genetic positive control.

~~**Figure 4. Workflow of micronucleus assay.** Mouse blood samples are collected into liquid heparin solution and fixed in cold methanol. Samples are washed in bicarbonate buffer, cells are pelleted and resuspended in buffer, before incubation in bicarbonate buffer containing FITC-conjugated anti-transferrin receptor (CD71) antibody and RNase. Samples are washed out of the incubation solution with bicarbonate buffer, and resuspended in buffer before adding propidium iodide (PI) and collecting 200 000 events by flow cytometry.~~

Figure 5. Analysis of micronucleated red blood cells by flow cytometry. a) The erythrocyte cell population is resolved from the rest of the cell population by region gate R1, created on side scatter logarithmic scale (SSC-Log) *versus* forward scatter linear scale (FSC-Lin). b) Single cells are isolated by region gate R2 on forward scatter width (FSC-W) *versus* FSC-Lin. c) Auto-fluorescent cells are excluded by gating on propidium iodide (PI) *versus* SSC (see also Suppl. Fig. 5). d) The gated cells (R1, R2 and R3) are analyzed

according to the intensity of fluorescence in the FITC channel (CD71-FITC) and the PI channel, and a quadrant gate is drawn to separate the four cell populations: normochromatic erythrocytes (NCE) are CD71-/PI-; reticulocytes (RET) are CD71+/PI-; micronucleated reticulocytes are CD71+/PI+ (MN-RET); while micronucleated normochromatic erythrocytes (MN-NCE) are CD71-/PI+.

Table 1. Troubleshooting ~~[AU: Editor has added in step numbers; please check.]~~

Step	Problem	Possible reason	Solution
2~~.~~4	-Samples tinged brown rather than bright red. -SSC *versus* FSC plot; reduction in FSC scattering i.e. population shifts left, and the cell populations are less well defined. (Suppl. Fig. 2d and f) and 3d-e)	- Samples have been fixed with warm fixative. - Samples have become too warm during preparation.	- Always use super-chilled methanol (-80˚C) to fix blood as warmer methanol temperatures change the size and granularity of blood cells and this results in overlap between anuclear red blood cells and nuclear white blood cells (SSC *versus* FSC plot). This causes an apparent elevation of the micronucleated-NCE population. Correct fixation is indicated by a bright red sample color in fixative (Suppl. Fig 2d)and a profile that fits the one shown in Fig. 5a. -Prepare samples on ice and centrifuge at 4˚C. - If the cellular origin of the PI-positivity is in question, we recommend performing fresh blood smears and staining with May-Grumwald Giemsa (Fig. 1b) or to cyto-spin methanol-fixed samples (e.g. from a duplicate 15 ml tube) onto slides and co-stain with DAPI (Fig. 1c and 1d).
13, 22, 23	Debris in bottom left hand corner of SSC *versus* FSC plot. (Suppl. Fig. 3b)	- Sample degradation. - Heparin solution made in water and not saline.	- Always fix the blood sample on the day of blood collection. The ideal profile fits the one in Fig. 5a.
17, 22, 23	CD71-FITC+ve-population predominates in Q2 rather than Q1 (See Suppl. Fig. 2b)	- PI binds RNA present in reticulocytes due to incomplete RNase treatment.	-Try a new batch of RNase and test using at least two RNase concentrations. -Reduce sample volume and increase buffer volume in assay.
2~~.~~1	Large variability in micronucleated-NCE (Q3) for mice of same genotype	-Incubation time in PI was very different between samples. (Suppl. Fig. 4b) -Mice are of a mixed genetic background. -Genotyping error.	-Add 1 µl PI to no more than 24 wells at a time before data acquisition and do not screen samples after 30 minutes incubation in PI. As with any good experimental practice, sample randomization in the plate should be used.

			-Increase sample number. -Re-genotype.
17, 22, 23	Spikes of events in Q2 (FITC *versus* PI plot) (Fig. 3c)	-Sample is too concentrated and/or collection speed is too high	-Reduce collection speed. -If spikes still occur in Q2 during acquisition, stop the acquisition and dilute the sample in bicarbonate buffer to ensure 1000-2000 events per second.
22, 23	Excessive number of events (>50-fold increase in total reticulocytes) in Q1 versus control mouse (RET; see Suppl. Fig. 1a)	Reticulocytosis	-Extreme reticulocytosis is likely to cause a false positive increase in the number of FITC-ve, PI+ve events (MN-NCE). Therefore it would be preferable to score the cells for the presence of a nucleus or micronucleus with a microscope after smearing methanol fixed blood onto slides and co-staining with DAPI (Fig 1c and 1d)[13].

The flow-cytometric quadrants (FITC *versus* PI plot) are defined as Q1: RET; Q2: MN-RET; Q3: MN-NCE; Q4: NCE.

Supplementary Figure 1. Severely increased red blood cell production (reticulocytosis) results in detection of nucleated and micronucleated (MN) normochromatic erythrocytes (NCE) in Q3. Blood obtained from C57BL/6J-*Apc*$^{Min/+}$ mice was processed according to the methods described herein. a) Representative flow-cytometric analysis for six week old *Apc*$^{-/min}$ mouse (left) and 16 week old *Apc*$^{min/+}$ mice (right). Note the change in % reticulocytes (RET) and %MN-NCE. During extremely high rates of erythropoiesis, such as that exhibited by severely anaemic, aged *Apc*$^{min/+}$ mice (right), the number of reticulocytes detected in the RET quadrant is vastly increased, however the process becomes error prone and the nucleus is not always expelled; both nucleated and micronucleated red blood cells are detected in the % MN-NCE quadrant, leading to a false-positive increase in %MN-NCE. b) Data shows %MN-NCE vs. %RET for *Apc*$^{min/+}$ mice (n=4) and *Apc*$^{+/+}$ mice (n=10). Linear regression analysis shows that there is a significant correlation between increased % MN-NCE and % RET (P<0.01). c) Dot plot showing the average %RET in male C57BL/6 mice (mean ± standard deviation: 1.623±0.209; n=20).

Supplementary Figure 2. Effect of RNase, flow rate and fixation. a) Blood sample obtained from male C57BL/6 mouse at 16 weeks of age, processed according to the methods described herein. b) The same sample was processed without the addition of RNase, which results in a shift of the FITC-conjugated transferrin receptor-positive (CD71-FITC+), propidium iodide negative (PI-) reticulocyte (RET) population over to the micronucleated-RET (MN-RET) quadrant. This is caused by PI binding to the RNA within the CD71-FITC+ RETs. c) Sample analyzed at a rate of 10000 events per second (vs. 1000 events in a) shows spikes of events in the CD71-FITC+ and PI+ quadrant (indicated by arrow head). d) The color of fixed samples can indicate whether samples have become too warm during preparation. Fresh blood samples were harvested and fixed in -80°C methanol in

duplicate or in room temperature (RT; 20-25°C) methanol. The blood fixed in -80°C methanol shows characteristic red color while the sample fixed in RT methanol shows brown color. e) Samples maintained for long periods of time in -80°C methanol show characteristic red color and are suitable for processing. f) The same samples (from image d) were either placed in a -80°C freezer or left at RT. After 30 minutes the sample that was maintained at -80°C showed the characteristic red color while the sample that was left at RT became brown in color; no detectable change in color was seen at the sample fixed in RT methanol.

Supplementary Figure 3. Indicators of incorrect sample processing. a) Correctly prepared sample. Blood sample obtained from male C57BL/6 mouse at 16 weeks of age, processed according to the methods described herein. The SSC-A vs. FSC-A plot (left) reveals correct separation of blood cell populations and minimal cell debris and the CD71-FITC vs. PI plot (right) reveals correct representation of populations within the quadrant gates. b) Slightly degraded blood sample. Blood sample left 5 days at 4˚C and fixed in -80˚C methanol. The SSC-A vs. FSC-A plot (left) reveals increased cell debris (black arrow) and incomplete separation of the cell populations. The gated populations plotted on CD71-FITC vs. PI plot (right) show no indication of degradation but there is a slight increase in MN-NCE (right). c) Very degraded blood sample. Blood sample left 3 weeks at 4˚C and fixed in -80˚C methanol. Both the SSC-A vs. FSC-A and CD71-FITC vs. PI plots are completely transformed. d) Blood sample fixed in warm methanol (room-temperature). The SSC-A vs. FSC-A plot (left) reveals increased cell debris (black arrow) and the CD71-FITC vs. PI plot (right) shows incorrect representation of populations. e) Blood sample fixed correctly (-80˚C methanol) but allowed to warm to room temperature (20-25°C) before processing. The SSC-A vs. FSC-A plot (left) reveals

increased cell debris (black arrow) and incomplete separation of the cell populations, and the CD71-FITC vs. PI plot (right) shows incorrect representation of populations.

Supplementary Figure 4. Effect of incubation time in propidium iodide (PI) and time in storage. a) Data shows %MN-NCE for replicate tubes of the same sample that were stored at -80˚C. Two tubes were processed at monthly intervals over a one-year period. Linear regression analysis shows that there is no significant difference in the detection of % MN-NCE up to one year after fixation (*P*=0.427). b) A single blood sample was added to each well of a 96-well plate and processed accordingly. After washing with buffer to remove the FITC-conjugated transferrin receptor (CD71-FITC)/RNase mix, samples were resuspended in buffer containing 1 mg/ml PI, and all 96 wells were immediately analyzed by flow-cytometry. Linear regression analysis shows that there is a statistically significant difference in the % micronucleated (MN)-normochromatic erythrocytes (NCE) obtained at the beginning of the plate *versus* those obtained at the end of the plate (**P*<0.001). This could be due to saturation of DNA with PI over time. The biological effect is small but to avoid potential experimental bias, samples should be randomized across the plate and we recommend adding PI to 24 wells at a time.

Supplementary Figure 5. Flow cytometric analysis of control samples and gating out autofluoresence. Unstained samples are used to centralise the main cell population within the side scatter (SSC) *versus* forward scatter (FSC) plot (data not shown). The main population is gated (erythrocytes), and within the propidium iodide (PI) *versus* FITC-conjugated transferrin receptor (CD71-FITC) plot a quadrant gate is drawn. Single-stain controls are then analysed to determine the correct voltage and

gain settings, which ensures the majority of events fall within the scale of the X and Y axes, and within the correct quadrant gate. a) Unstained control sample and b) CD71-FITC alone. Auto-fluorescence can be detected with samples stained with PI alone, either within the transferrin receptor FITC-conjugated (CD71-FITC) channel (c; indicated by arrowhead), or within the higher logarithmic scale (Log) of PI channel (d; indicated by arrowhead). To remove the effect of auto-fluorescence, Region gate R3 is created within the PI *versus* side scatter (SSC) plot (d).

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