Chapter 6

Isolation of Plant Nuclei at Defined Cell Cycle Stages Using EdU Labeling and Flow Cytometry

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Abstract

5-Ethynyl-2′-deoxyuridine (EdU) is a nucleoside analog of thymidine that can be rapidly incorporated into replicating DNA in vivo and, subsequently, detected by using "click" chemistry to couple its terminal alkyne group to fluorescent azides such as Alexa Fluor 488. Recently, EdU incorporation followed by coupling with a fluorophore has been used to visualize newly synthesized DNA in a wide range of plant species. One particularly useful application is in flow cytometry, where two-parameter sorting can be employed to analyze different phases of the cell cycle, as defined both by total DNA content and the amount of EdU pulse-labeled DNA. This approach allows analysis of the cell cycle without the need for synchronous cell populations, which can be difficult to obtain in many plant systems. The approach presented here, which was developed for fixed, EdU-labeled nuclei, can be used to prepare analytical profiles as well as to make highly purified preparations of G1, S, or G2/M phase nuclei for molecular or biochemical analysis. We present protocols for EdU pulse labeling, tissue fixation and harvesting, nuclei preparation, and flow sorting. Although developed for Arabidopsis suspension cells and maize root tips, these protocols should be modifiable to many other plant systems.

Key words 5-Ethynyl-2′-deoxyuridine, Flow cytometry, Nuclei sorting, Arabidopsis, Maize, Percoll gradient, Cell cycle, DNA replication, Chromatin

1 Introduction

To investigate cell cycle-related processes, it is critical to be able to separate cells in distinct stages of the cell cycle for analysis. Mechanisms governing key cell cycle processes like chromosome segregation and DNA replication can then be studied. To do this, cells preparing to enter replication or go through cell division must be distinguished from actively replicating cells with the same DNA content. To this end, many types of nucleoside precursors have been used as labels for DNA synthesis over the years. Currently, the most efficient and versatile DNA precursor for

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fluorescent labeling is 5-ethynyl-2′-deoxyuridine (EdU), a nucleoside analog of thymidine. EdU, which has become the new standard for labeling newly replicating DNA, has been used to accomplish a wide range of experimental goals in diverse plant species (reviewed in [1]). Darzynkiewicz et al. [2] reviewed the benefits of using EdU in place of other precursors such as 5-bromo-2′-deoxyuridine (BrdU). In particular, eliminating the denaturation step that is necessary for antibody detection of BrdU helps maintain DNA and protein structural integrity and enhances structural preservation [2].

After EdU is incorporated into DNA, the terminal alkyne group can be coupled to a fluorescent azide such as Alexa Fluor 488 (Alexa-488) [3] through a process called copper(I)-catalyzed azide-alkyne cycloaddition or "click chemistry" [4]. This reaction is quite robust, and can be carried out on fixed or unfixed biological material. Labeled material can be used for flow cytometry, microscopy, fluorescence in situ hybridization (FISH), and immunochemistry in many plant tissues [5]. However, the few studies that have combined EdU labeling with flow cytometry were analytical in nature, and did not use flow sorting to enrich for plant nuclei at specific stages in the cell cycle. We have developed a method that uses flow sorting to prepare pure populations of unlabeled G1 and G2/M nuclei separate from EdU/Alexa-488-labeled, S-phase nuclei. This approach for staging nuclei allows us to start with an asynchronous population of nuclei and avoid artifacts sometimes associated with cell cycle synchronization procedures, such as sucrose starvation or incubation with nucleotide synthesis inhibitors [6].

Flow cytometric analysis of plant nuclei was originally made possible by the development of the widely used tissue chopping method introduced by Galbraith and colleagues [7]. They found that chopping with a razor blade mechanically releases nuclei from plant cells with a minimum of shear stress and structural disruption. The resulting homogenate may include a large amount of cellular debris, but when debris is reduced by filtration; such preparations can be analyzed on a flow cytometer. Modifying this original method, we found that nuclei can be released from fixed, frozen plant cells or tissues using a commercial blender or food processor by gentle blending in short bursts in cold cell lysis buffer. This modification allows us to dramatically scale up the amount of material we can process for flow sorting. Different plant species and tissues yield different levels of unwanted cellular debris during this process, and the nuclei isolation procedures must be optimized carefully. High levels of debris in a sample can greatly reduce the efficiency of sorting.

Several plant studies have described flow sorting of fresh or fixed nuclei based on DNA content [8–10]. Plant protoplasts have also been sorted based on their expression of GFP or other

fluorescent proteins [11–14]. However, protoplast preparations are not typically used for experiments where DNA content needs to be accurately estimated, because the DNA signal is often confounded by nonspecific binding of DNA fluorochromes, autofluorescence from plant pigments, and the non-symmetric position of nuclei within plant cells, which can alter light scattering properties during sorting [15].

The protocol presented here highlights the combination of EdU pulse labeling and preparative sorting of nuclei isolated from fixed plant cells or tissues. By adding a second parameter in addition to DNA content for the flow sorting—e.g. the fluorescence of Alexa-488 coupled to EdU as a measure of DNA synthesis, we can distinguish nuclei in different stages of the cell cycle with unlabeled G1 and G2/M nuclei separated from the arc of EdU-labeled, S-phase nuclei (Fig. 2 panels c and g). Importantly, G1 and G2/M nuclei with 2C and 4C DNA content, respectively, are readily resolved from nuclei in very early or very late S phase, which have similar DNA content. We discuss flow cytometry parameters and conditions for sorting plant nuclei using Arabidopsis suspension cells or maizeroot tips as starting material. Arabidopsis suspension cells provide a relatively homogeneous model system, while maize roots offer an opportunity to explore developmental regulation in the context of a whole tissue [1]. By describing methods of preparing and sorting nuclei from these two very different plant systems, our hope is to show how the protocols described here can be adapted to other plant systems.

2 Materials

All chemicals are reagent grade, unless otherwise noted.

2.1 Plant Material and Growth

2.1.1 Maize Seedlings

1. Plant material: *Zea mays* cv B73 seeds. The total number of seeds required should be determined empirically based on the germination rate and the amount of material required for the downstream analysis. A single experiment typically requires 400–800 maize seedlings.
2. 150 mL 10 % bleach containing 1–2 drops of Tween 20.
3. Supplies: magnetic stir plate, stir bar, large plastic or glass container, small fish tank bubbler, Magenta™ boxes, paper towels.
4. Equipment: growth chamber.

2.1.2 Arabidopsis Cells

1. Plant material: Arabidopsis suspension cells (Col-0, ecotype Columbia).
2. 2,4-Dichlorophenoxyacetic acid (2,4-D): 100 mg in 10 mL of 95 % ethanol, store at −20 °C.

3. Arabidopsis culture medium: 3.2 g/L of Gamborg's B5 basal medium with minimal organics, 3 mM MES, 3 % sucrose, 1.1 mg/L 2,4-D, pH 5.8, autoclave and store at 4 °C.
4. Supplies: 250-mL baffled flasks, serological pipettes.
5. Equipment: laminar flow hood, refrigerated console shaker with a light bank.

2.2 Labeling Newly Replicated DNA In Vivo and Harvesting Tissue

1. 5-Ethynyl-2′-deoxyuridine (EdU) (Life Technologies): 40 mM solution in dimethyl sulfoxide (DMSO), store at −80 °C.
2. 1× Phosphate buffered saline (PBS).
3. 16 % Paraformaldehyde (EM Grade).
4. 2 M Glycine, filter-sterilized.
5. Liquid nitrogen.

2.2.1 Maize Seedlings

1. Supplies: #10 scalpel, fine tipped forceps, Petri dishes, 2-mL microcentrifuge tubes.
2. Equipment: bench-top orbital shaker, vacuum pump attached to a desiccator.

2.2.2 Arabidopsis Cells

1. Supplies: 50-mL conical tubes.
2. Equipment: refrigerated swinging bucket centrifuge.

2.3 Isolation of Nuclei

1. Cell lysis buffer (CLB): 15 mM Tris (pH 7.5), 2 mM Na_2EDTA (pH 8), 80 mM KCl, 20 mM NaCl, 0.1 % Triton X-100. Adjust pH to 7.5 then add 15 mM 2-mercaptoethanol (buffer modified from LB01 in [15]).
2. Complete or Complete Mini protease inhibitor cocktail tablets (Roche).
3. Supplies: Miracloth, small plastic funnel.
4. Equipment: commercial blender (like Cuisinart, model SPB-7) or food processor (like Cuisinart mini-prep).

2.4 Percoll Gradient Purification (Optional)

This optional procedure is modified from [16], and is only necessary when the percentage of nuclei is very low in relation to the percentage of unwanted debris in the sample.

1. Gradient buffer (5×): 2.5 M hexylene glycol, 25 mM PIPES-KOH (pH 7.0), 50 mM $MgCl_2$, 25 mM 2-mercaptoethanol, and 5 % Triton X-100. 5× gradient buffer can be prepared as a stock solution without 2-mercaptoethanol and stored at 4 °C. Add 2-mercaptoethanol immediately before use.
2. Gradient buffer (1×): 0.5 M hexylene glycol, 5 mM PIPES-KOH (pH 7.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 1 % Triton X-100. Dilute to 1× from the 5× stock buffer and add 2-mercaptoethanol immediately before use.

3. Extraction buffer (1×): 2.0 M hexylene glycol, 20 mM PIPES-KOH (pH 7.0), 10 mM $MgCl_2$, and 5 mM 2-mercaptoethanol.
4. 30 % Percoll: 100 % Percoll diluted in 5× gradient buffer and sterile H_2O to achieve a final concentration of 1× gradient buffer.
5. 80 % Percoll: 100 % Percoll diluted in 5× gradient buffer and sterile H_2O to achieve a final concentration of 1× gradient buffer.
6. Supplies: 50-mL glass Corex tubes with screw cap.

2.5 Clicking EdU to Alexa Fluor 488

1. Click-iT®EdU Alexa Fluor® 488 Imaging Kit (Life Technologies).
2. Modified CLB: 15 mM Tris (pH 7.5), 80 mM KCl, 20 mM NaCl, 0.1 % Triton X-100, pH 7.5.
3. DAPI stock solution: 1 mg/mL in sterile H_2O.
4. CLB containing 2 μg/mL DAPI.
5. Equipment: refrigerated microcentrifuge.

2.6 Flow Cytometric Sorting

1. 1× Sodium Chloride-Tris-EDTA (STE) buffer: 10 mM Tris–HCl (pH 7.5), 1 mM EDTA (pH 8), 100 mM NaCl, pH 7.5.
2. Laser alignment beads suitable for the flow sorter and particle size to be analyzed. For the BD InFlux 3–3.4 μm and 8.1–12 μm Ultra Rainbow Fluorescent Beads (Spherotech) are one option.
3. Nuclei collection buffer suitable for the downstream application (*see* **Note 14** and Table 1).
4. CellTrics® 20 μm nylon mesh filters (Partec).

Table 1

Collection buffers and post-sort nuclei handling

Downstream application	Collection buffer	Immediate post-processing	Method reference(s)
DNA-IP or other DNA application	STE buffer	Freeze in STE or immediately process	[18]
ChIP	2× Extraction buffer 2	Centrifuge, extract chromatin	[19]
Microscopy/FISH	2× CLB without 2-mercaptoethanol	Store protected from light at 4 °C	[1], Bass et al. in preparation
Sort reanalysis	CLB-DAPI	None	–

5. Supplies: 5-mL round-bottom polypropylene tubes, microscope slide.
6. Equipment: fluorescence microscope, flow sorter equipped with a 355 nm UV laser and a 488 nm sapphire laser.

3 Methods

3.1 Plant Material and Growth

3.1.1 Maize Seedlings

1. Imbibe the maize seeds overnight in constantly moving water with aeration. To do this, set up a large container with a stirbar and a fishtank bubbler, and fill it with diH_2O.
2. Surface-sterilize the seeds by adding the 10 % bleach with Tween 20 and gently shake for 10 min. Rinse with sterile H_2O three times.
3. Germinate the seeds in autoclaved magenta boxes containing several layers of paper towel dampened with sterile H_2O. Place 10–12 seeds per box and place the boxes in a growth chamber set to 28 °C with continuous dim light (~500 lux) for 3 days.

3.1.2 Arabidopsis Cells

1. Arabidopsis suspension cells are grown in 250-mL baffled flasks containing 50 mL medium in a refrigerated console shaker set to 23 °C and 160 rpm with constant light (~2000 lux). Propagate the cells every 7 days by transferring a 6 mL aliquot to a flask with 50 mL fresh, pre-warmed medium. To maximize EdU incorporation, cells have to be in the logarithmic phase of the growth curve [8]. To achieve this, 25 mL of 7-day cells are diluted 1:1 in an equal volume of fresh, pre-warmed medium and grown for 16 h before adding EdU.

3.2 Labeling Newly Replicated DNA In Vivo and Harvesting Tissue

3.2.1 Maize Seedlings

1. Place the 3-day-old seedlings in a large container with 100–300 mL sterile H_2O. Swirl to rinse and pour off the water.
2. Depending on the number of seedlings, add 100–300 mL of sterile H_2O containing a final concentration of 25 μM EdU, making sure the roots are covered in the water. Incubate the seedlings for the desired pulse-labeling time (*see* **Note 1**) with gentle shaking (65 rpm) on a bench-top orbital shaker at room temperature in the dark.
3. Pour off the EdU solution and rinse the seedlings three times with sterile H_2O, leaving some of the last rinse in the container to keep the roots moist.
4. Prepare a solution of 20 mL 1 % paraformaldehyde in 1× PBS.
5. Dissect the desired root tip segment in a Petri dish using a #10 scalpel and fine-tipped forceps. Transfer the cut segments to a small beaker or tube containing 20 mL of 1 % paraformaldehyde and fix for 15 min, with the first 5 min under vacuum. The length of time cutting a batch of root segments should be minimized as much as possible.

6. Add 1.33 mL of 2 M glycine (final concentration 0.125 M) to quench the fixation, and incubate for 5 min under vacuum.
7. Wash the root segments three times with ~20 mL 1× PBS and remove the liquid completely with a pipet after the final wash. Transfer the roots to a 2-mL tube and snap-freeze in liquid nitrogen. Store at −70 °C.

3.2.2 Arabidopsis Cells

1. Add 12.5 μL of 40 mM EdU (final concentration 10 μM) to each flask containing 50 mL of cell culture in logarithmic phase (the 1:1 diluted culture, grown for 16 h).
2. Allow cells to incorporate EdU for the chosen amount of time (*see* **Note 1**) by incubating in the console shaker.
3. Add 3.33 mL of 16 % paraformaldehyde to each flask (final concentration 1 %) and place in the console shaker for 10 min.
4. Add 3.55 mL of 2 M glycine (final concentration 0.125 M) to quench fixation, and incubate for 5 min.
5. Pour the contents of each flask into a 50-mL conical tube.
6. Centrifuge at 200 × *g* for 3 min and remove the supernatant.
7. To wash the cells, dilute to 50 mL with 1× PBS and gently invert the tube.
8. Centrifuge at 200 × *g* for 3 min and remove the supernatant.
9. Repeat **steps 7** and **8** for a total of three washes.
10. After the final wash, pipette off the supernatant and remove the residual PBS by pressing the tip of a pipette against the bottom of the conical tube and slowly aspiring until the cells are dry and there is no visible liquid.
11. Snap freeze the tube in liquid nitrogen and store at −70 °C.

3.3 Isolation of Nuclei

Nuclei are isolated from fixed, frozen cells or tissues by chopping the frozen pellet in a blender or food processor in cold CLB. All steps should be done on ice or at 4 °C. Additionally, the nuclei must be re-suspended very well after every centrifugation to ensure that large clumps of aggregates and debris do not form (*see* **Note 3**).

1. Set up a double layer of Miracloth in the top of a small plastic funnel. Place the blender or food processor inside a cold room or fridge before starting. Set a large refrigerated swinging bucket centrifuge to 400 × *g* at 4 °C and a refrigerated micro-centrifuge to 200 × *g* at 4 °C.
2. Make fresh CLB and keep on ice. If using, dissolve a Complete or Complete Mini protease inhibitor cocktail tablet in the water used to make the buffer (*see* **Note 13**). Add an appropriate amount of CLB to the blender or food processor (*see* **Note 2**).
3. Take the sample tube out of the −70 °C freezer, and tap the tube until the cell or tissue pellet is released from the bottom

of the tube. Two frozen pellets can be chopped together in large-scale experiments. If the pellet does not release from the tube, a small amount of CLB can be added and forceps inserted to break the pellet.

4. Transfer the frozen pellet to the blender or food processor. Grind the pellet on a low speed (low or chop) for 15–30 s at a time with 15–30 s breaks. Do this two to six times depending on the length of the chop and the tissue and species used.
5. Allow the cellular homogenate to incubate in the blender or food processor for 5 min at 4 °C to facilitate nuclei release from the cells.
6. Place the Miracloth plus funnel over a 50-mL conical tube or small beaker on ice. Moisten the Miracloth by adding 5–10 mL CLB and discard the flow-through.
7. Swirl to mix the cellular homogenate and filter through the Miracloth into the tube or beaker. Allow the homogenate to filter through for several minutes, then gently press on the Miracloth to remove excess buffer.
8. Dispense the filtered homogenate into 2–4 pre-chilled 50-mL conical tubes (each containing 15–20 mL) and centrifuge at 400 × *g* for 5 min at 4 °C. Pipette off the supernatant being careful not to disturb the white nuclei pellet.
9. Proceed immediately to the optional Percoll gradient purification (Subheading 3.4) or to the click reaction (Subheading 3.5).

3.4 Percoll Gradient Purification (Optional)

Several sample preparation conditions may need to be tested and analyzed on the flow cytometer to determine the optimal set up for each sample type. Preliminary flow cytometer results can determine the relative percent of the sample that is nuclei compared to small or large debris, and further improvements should be made to the blending conditions or filtering if possible. If the nuclei isolation cannot be improved further, you can consider using a density gradient purification step to remove some debris after nuclei isolation. An optional Percoll gradient purification protocol (adapted from [16]) is presented here (*see* **Note 4**).

1. Prepare 15 mL of 1× gradient buffer and 30 mL 1× extraction buffer per gradient. Also prepare 7 mL 80 % Percoll and 19 mL 30 % Percoll per gradient and keep all buffers on ice.
2. The number of gradients needed per sample must be empirically determined. Use a serological pipette with a bulb or an electric pipettor at low speed to layer each gradient in a glass Corex tube. First, add 12 mL of the 30 % Percoll layer, then sub-layer 6 mL of 80 % Percoll, and put on ice (see diagram in Fig. 1).

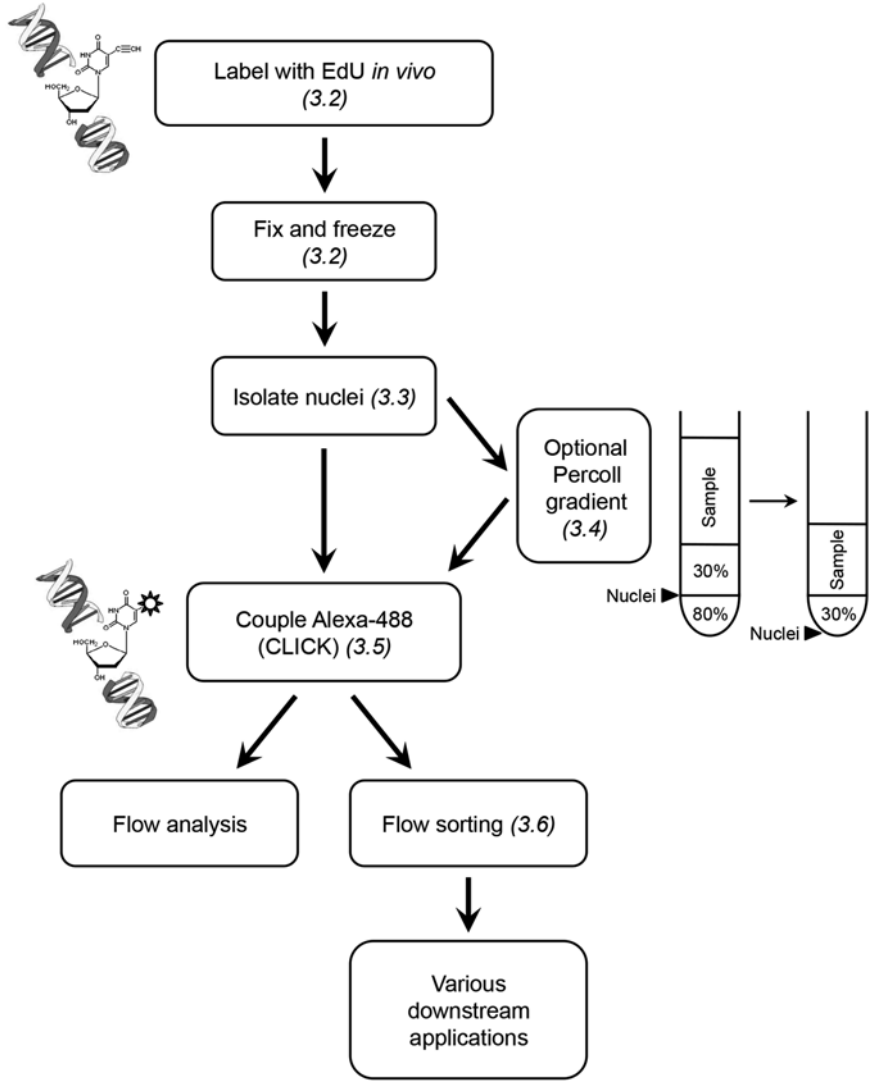

Fig. 1 Flow chart of the protocol to EdU pulse label and prepare nuclei for flow sorting

3. Re-suspend the nuclei pellet in each 50-mL conical tube with 2 mL 1× extraction buffer. To do this, gently pipet up and down at least 50 times for each tube. Combine the nuclei into one tube and bring the final volume up to 30 mL in 1× extraction buffer.
4. Using a slow, constant speed, add the re-suspended nuclei to the top of the gradient. Do this by putting the pipette against the side of the tube and slowly releasing the sample until there is about half an inch in depth, and then the sample can be added in the center of tube.

5. Centrifuge at 1500 × *g* for 30 min at 4 °C.
6. Take off the top layer of gradient buffer with a glass pipette. Do not allow back flow, which could disrupt the gradient.
7. Use a glass pasture pipette to carefully remove the white nuclei layer, and transfer to another cold 50-mL glass Corex tube.
8. Re-suspend the nuclei well in 1× gradient buffer, to a total volume of 10 mL. Then slowly underlay with 6 mL of 30 % Percoll.
9. Centrifuge at 1500 × *g* for 10 min at 4 °C. Remove the supernatant (buffer and Percoll), the nuclei are pelleted at the bottom of the tube.
10. Wash the nuclei pellet with 3–5 mL of 1× extraction buffer. To do so, re-suspend the nuclei well, centrifuge at 1500 × *g* for 10 min at 4 °C and remove the supernatant.
11. Proceed immediately to the click reaction (Subheading 3.5).

3.5 Clicking EdU to Alexa Fluor 488

Before the click reaction, the nuclei must be washed in a buffer without 2-mercaptoethanol and Na_2EDTA, so the reaction can proceed efficiently.

1. Wash each 50-mL sample tube with 2 mL Modified CLB and re-suspend the white nuclei pellet very well by pipetting 50 times for each tube. Transfer the re-suspended nuclei into two 2-mL microcentrifuge tubes.
2. Centrifuge at 200 × *g* for 5 min at 4 °C to pellet nuclei. Remove the supernatant by pipetting.
3. Prepare the Click-iT® reaction cocktail according to the kit instructions (*see* **Note 5**).
4. Add the Click-iT® reaction cocktail and re-suspend the nuclei well. Depending on the amount of reaction cocktail used, split the sample between sufficient 2-mL tubes to maintain ~1 mL in each tube. Incubate 30 min at room temperature in the dark.
5. Centrifuge at 200 × *g* for 5 min at 4 °C and remove the supernatant.
6. Depending on the amount of reaction cocktail used, add twice that volume of CLB to wash out the reaction cocktail, and re-suspend the nuclei very well. Centrifuge as before, and remove the supernatant.
7. Re-suspend the nuclei pellet in an appropriate volume of CLB-DAPI (*see* **Note 6**) depending on nuclei yield. Pipet the sample gently ~100 times, but try to avoid making bubbles.
8. Check a small aliquot of the nuclei on a fluorescence microscope to verify nuclei quality and DAPI and Alexa-488 fluorescence (DAPI needs at least 5 min for staining).

9. Just before flow cytometric analysis and sorting, filter the nuclei suspension through a 20-μm nylon mesh filter into a 5-mL round-bottomed polypropylene tube to remove large debris.

3.6 Flow Cytometric Sorting

Flow sorting may take place in consultation with a flow-core facility that will have specific recommendations and settings. Settings for an InFlux (BD Biosciences, originally Cytopeia) are described here, but these settings should be considered as suggested values only. The specific settings will need to be determined empirically based on the experimental goals, sample type, and flow sorter.

1. Turn on and prepare the flow sorter. A 70-μm nozzle tip is used with 16 psi sheath pressure. This gives stable droplet formation with a droplet delay of 17.6, and a piezo amplitude of 1–3 V. However, machine settings change some from day to day, and over the life of the instrument.
2. Using a forward angle light scatter (FSC) trigger (*see* **Note 7**), prepare the software to measure FSC, 90° angle side scatter (SSC), and emission at 460/50 nm for DAPI fluorescence and at 530/40 nm for Alexa-488 fluorescence.
3. Begin running a sample tube containing the nuclei preparation and adjust the sample pressure to achieve an event rate of 3000–5000 events/s (*see* **Note 8**).
4. First, create dotplots of FSC vs. SSC, FSC vs. DAPI fluorescence, and SSC vs. DAPI fluorescence (Fig. 2 panels a and e). Additionally, a FSC threshold or cutoff should be set empirically to minimize the amount of small debris in the analysis, without excluding the particles of interest. Apply the photomultiplier tube (PMT) voltage settings so that the measured events are centered in the plot. However, because plant tissue homogenates are inherently noisy, and the nuclei may be a small percentage of the total particles (*see* **Notes 3** and **4**), you may need to reduce the FSC or SSC voltages so that the measured events are centered more in the bottom third of the plot.
5. In the FSC vs. DAPI fluorescence and SSC vs. DAPI fluorescence plots, distinct populations should be visible for 2C, 4C and larger ploidy nuclei on the DAPI axis. Create a univariate histogram of DAPI fluorescence to see this clearly (Fig. 2 panels b and f). The coefficient of variation (CV) should be calculated for the G1 peak, which ideally should be below 5 % (*see* **Note 9**).
6. Next, create a dotplot of Alexa-488 fluorescence (530/40 nm—Height) vs. DAPI fluorescence (460/50 nm—Height). Apply the PMT voltage settings so that an arc of Alexa-488-labeled nuclei in S phase can be seen above the unlabeled G1

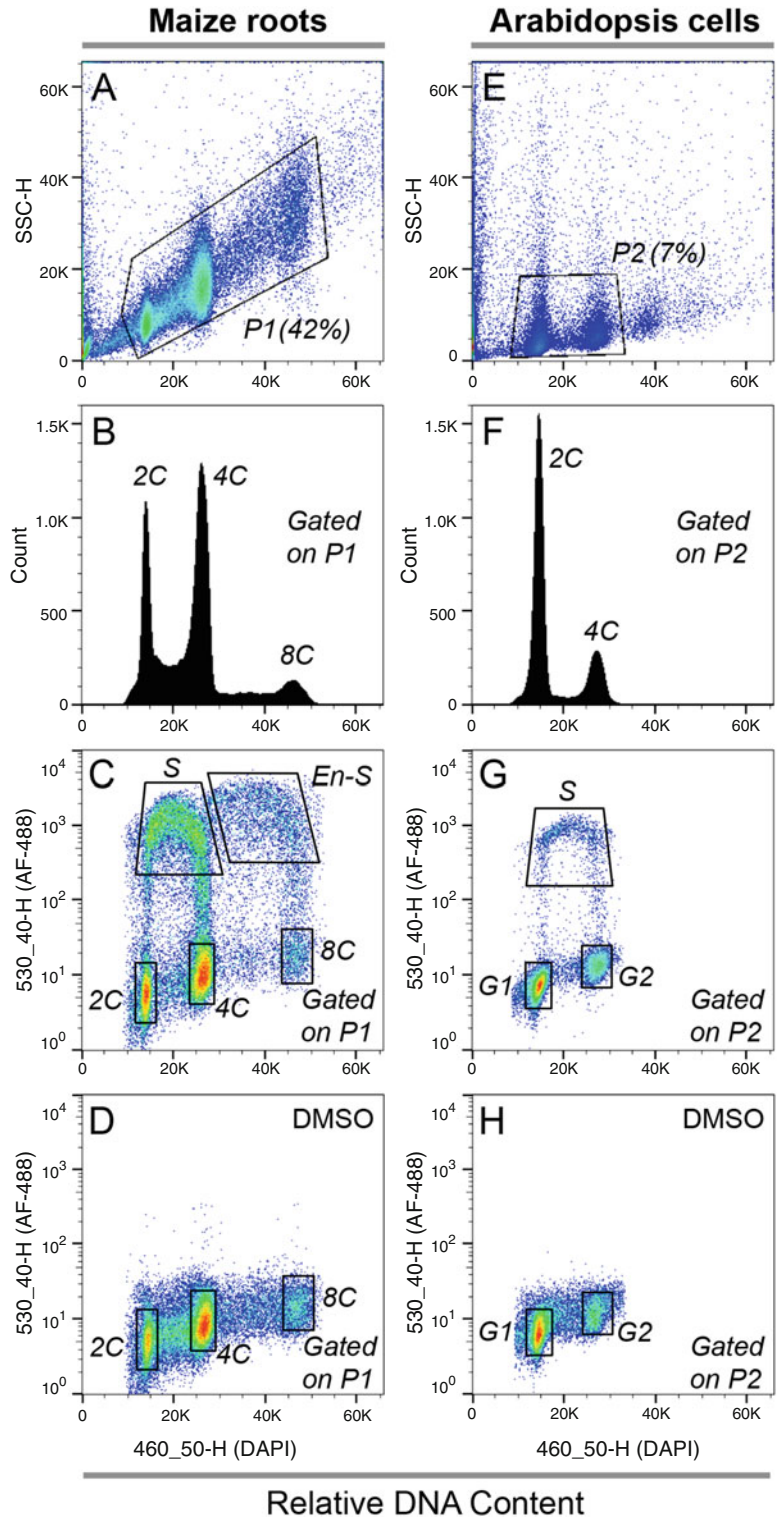

Fig. 2 Flow cytometric analysis and sorting of nuclei prepared from maize roots and Arabidopsis cells. (**a–d**) Intact maize roots were pulse-labeled with EdU for 20 min. The first 3 mm of the root tip was then dissected, fixed, and frozen. (**e–h**) Arabidopsis suspension cells were pulse-labeled with EdU for 30 min, followed by

and G2/M nuclei. Depending on the tissue type used, there may be multiple arcs for several rounds of endoreduplication (*see* Fig. 2 panels c and g).

7. Place sorting gates to divide the cell cycle populations as desired (*see* **Note 10**).
8. Set the sort mode based on the experimental needs and abundance of the nuclei to be sorted. Single-droplet purity mode can be used to achieve a sort efficiency of 85–95 %.
9. Validate the sort parameters and gate placement by sorting 100 nuclei onto a microscope slide and observing and counting them on a fluorescence microscope. It is also important to reanalyze a small amount of sorted nuclei to further confirm sorting parameters and to determine the sort purity (*see* **Note 12**).
10. The amount of nuclei to be sorted for an experiment should be empirically determined for each plant species and downstream application (*see* **Note 13**). Nuclei preparations are often split between several round-bottomed 5-mL tubes that must be kept on ice and protected from light while sorting. Use the cooling function on the flow sorter to keep both the sample tube and the nuclei collection tubes chilled.
11. Depending on the downstream application, the collection buffer for sorted nuclei and handling after sorting will vary (*see* **Note 14** and Table 1).

4 Notes

1. *EdUpulse length*. Depending on the experimental goals, the length of the EdU pulse should be selected carefully. In many cases, knowing the approximate length of S phase in the material under study will allow a more informed decision on pulse length. We have found that a 5 min EdU pulse is detectable by

Fig. 2 (continued) fixation and freezing. Nuclei were prepared and analyzed by flow cytometry. (**a, e**) Typical bivariate plots of side scatter (SSC) and relative DNA content (measured by DAPI fluorescence in the 460 ± 50 nm detection range). Nuclei are gated on SSC to exclude small and large cellular debris as well as damaged nuclei from further analysis and sorting. The SSC gate can be drawn to include endocycling nuclei, as depicted for maize roots in panel (**a**), or exclude endocycling and anueploid nuclei, as depicted for Arabidopsis cells in panel (**e**). (**b, f**) The corresponding univariate histograms of relative DNA content, which only include events within the parent SSC gates (P1 in panel (**a**), or P2 in panel (**e**)). (**c, g**) Bivariate plots of EdU incorporation (measured by Alexa-488 fluorescence in the 530 ± 40 nm detection range) and relative DNA content show nuclei that are actively replicating their DNA as an arc above the G1 and G2/M populations. Sorting gates can be placed to purify nuclei from different cell cycle phases, including 2C/G1, 4C/G2, 8C, S, and endocycling S (En-S). (**d, h**) Samples were prepared as described above, except roots or cells were treated with DMSO in place of the EdU. Mock-labeled control samples are useful for appropriate sort gate placement

flow cytometric analysis, but a 10–15 min pulse is more useful to achieve the separation of the S-phase arc needed for sorting. For many purposes longer pulses (30 min–1 h) are more practical, but the appropriate time depends on many factors, including the desired cell cycle population, percentage of cells in S phase in the tissue, genome size, sorting yield, sensitivity of the analysis, and the downstream application.

2. *Blending optimization.* The amount of CLB will depend on the specific blender or food processor, and the position of the blades. We routinely use ~80 mL CLB in a large Cuisinart blender for Arabidopsis cell pellets, and ~35 mL CLB in a Cuisinart mini-prep food processor for maize roots. Use one of the lowest settings (low or chop) for short intervals with resting periods to allow the liquid to collect back in the bottom of the jar. For maize root tips, we use 5–6 15-s intervals with 15-s rests. For Arabidopsis cell pellets, we use two 30-s intervals with a 30-s rest.
3. *Nuclei handling.* In some plant nuclei samples, the percentage of the target population will be small. This percentage will decrease further if the tissues are blended too much, nuclei isolation conditions are not optimal, or there are large aggregate clumps. It is extremely important to re-suspend the nuclei well at each and every step during sample preparation to avoid forming these clumps. Large aggregate clumps will reduce the yield of nuclei that can be sorted, and can clog the instrument sample line or nozzle tip. Following every centrifugation, pipet gently up and down at least 50 times. Additionally, some tissues and cell types result in more debris and small particles than others. If the nuclei preparation cannot be improved further by technical optimization, consider using a Percoll gradient purification step (*see* **Note 4**).
4. *When to use a Percoll density gradient.* In a large-scale experiment, sorting a sample with a small percentage of target nuclei can be very time-consuming and, for this reason, can become the limiting step. Particularly for Arabidopsis cells, the main issue is often the presence of debris particles that are smaller than nuclei, but not so much smaller that they can be effectively excluded by setting a threshold cutoff on the flow sorter. The debris particles are counted as events, which can slow down the sort significantly. To remove some of the debris before sorting, a further Percoll gradient purification step can be performed.
5. *Click-iT® reaction cocktail.* The amount of Click-iT® reaction cocktail to use for a particular starting tissue amount should be empirically determined. As a starting place, we recommend 0.5 mL of reaction cocktail for a 250-mL flask of 7-day split

Arabidopsis cells, and 2 mL cocktail for the roots from ~250 maize seedlings.

6. *Final sample volume*. The amount of CLB-DAPI used for the final re-suspension of nuclei is variable and can depend on the efficiency of nuclei release, any losses that occurred during handling (especially if Percoll gradient purification is used), and the optimal sample rate of the flow sorter (*see* **Note 8**).
7. *Event triggering*. Events can be triggered on either FSC or SSC. Some find that triggering on SSC in plant preparations can be less noisy [12]. In our experience with plant nuclei preparations from fixed tissue, there was not much benefit from triggering on SSC, and therefore chose to trigger on the traditional FSC. Some instruments may allow triggering on UV fluorescence, depending on the specific configuration of the lasers, which may be advantageous for samples that have a large amount of non-fluorescent debris.
8. *Event rate and sort rate*. The optimal event rate will depend on the type of flow sorter, the sheath pressure, and quality of the sample preparation. The relative "clumpiness" of the sample will increase the possibility of clogs in the sample line or nozzle, and can decrease the sort efficiency (number of sorted events/(sort aborts + sorted events) × 100). We typically use an event rate between 3000 and 5000 events/s. The sort rate, or rate that nuclei from each sort gate will accumulate, will vary depending on the percentage of the targeted population.
9. *Sample quality control*. Ideally, the CV of the G1 peak should be below 5 % [17]. However, we have experienced peaks with a CV of ~5–6.5 %, that still have good sort purity upon reanalysis, and yield good downstream results. Given that the end goal of our approach is to sort different cell cycle populations for further analysis, this is likely not an issue. Slightly larger CVs may be unavoidable because of the large-scale sample preparations needed for sorting. If the CV of the G1 peak is wider than expected, it is likely caused by poor sample quality. Checking the nuclei preparation on a fluorescence microscope will help identify if the sample has large aggregate clumps or if nuclei appear damaged from over-blending, or over-centrifugation. The plot of SSC vs. DAPI fluorescence is also useful to determine if the nuclei have irregular light scattering properties, which can indicate damaged or misshapen nuclei (*see* Fig. 2 panels a and e).
10. *Sort gates*. If necessary, hierarchical gates can be used to eliminate cellular debris as well as damaged or misshapen nuclei based on SSC (*see* Fig. 2 panel a and e). Then gating of the cell cycle is done only on the subsequent daughter population. The SSC gate can be drawn to include endocycling nuclei, as is

depicted for maize roots in panel a, or to exclude endocycling and anueploid nuclei, as is depicted for Arabidopsis cells in panel e. Sort gates can be set in various ways depending on the experimental goals (for example, *see* Fig. 2 panels c and g). Negative controls should also be used to verify the gating strategy is appropriate (*see* **Note 11** and Fig. 2 panels d and h).

11. *Negative controls for sort gating*. When optimizing the EdU pulse label, it is also important to run negative control samples for each treatment and label time. In the case of a short label, where a weaker signal is expected, negative controls are critical for setting sort gates. Usually, the most useful control for gating is a sample that is mock-labeled with DMSO, and then the click reaction and all other procedures are done in parallel to the experimental sample (Fig. 2 panels d and h).
12. *Sort reanalysis*. It is important to reanalyze a small amount of the nuclei to determine the sort purity and to ensure that sort settings and gates are appropriate. For reanalysis purposes, sort at least 12,000–15,000 nuclei into a small amount (~200 μL) of CLB-DAPI. In our experience, DAPI is more susceptible to bleaching than Alexa-488, however re-staining in CLB-DAPI alleviates most of the DAPI bleaching. Save data files to analyze the percentage of nuclei that fall back in the original sort gate. Some population spreading is inherent due to the fact that nuclei may not be exactly round and will pass through the laser beam the second time in different orientations.
13. *Sort yield and timeframe*. The number of nuclei sorted (sort yield) will vary based on losses during nuclei isolation and other purification steps, sorting parameters, and the size and number of gates. The number of nuclei needed for a downstream application will also vary. Preliminary sorts are required to determine the sort yield and timeframe. For longer sorts, the nuclei preparation can be done the day before a sample is run on the machine, as long as a Complete protease inhibitor cocktail tablet is used in the CLB buffer. The sample should be stored overnight at 4 °C and protected from light. **Steps 7–9** of Subheading 3.5 should be completed the morning of the sort.
14. *Nuclei handling aftersorting*. Depending on the volume of nuclei to be sorted, add 200 μL–1 mL of collection buffer (*see* Table 1 for buffer options for various downstream applications) to a 2-mL collection tube. Other collection tubes and plate formats may also be used, if the flow sorter allows. The sorted droplets of sheath fluid (STE buffer) containing the nuclei will dilute the collection buffer, necessitating the use of a 2× buffer in some cases. For applications where a large quantity of nuclei are needed, multiple tubes of sorted nuclei may need to be combined and centrifuged to remove excess buffer

(850 × *g* for 10 min at 4 °C) before proceeding. The nuclei pellet will likely be invisible at this stage, so leave ~0.5 mL of supernatant over the pellet. If the volume of sorted nuclei is sufficiently small, the sample can be stored at 4 °C (for microscopy), frozen at −70 °C (for DNA applications), or immediately processed to extract DNA or chromatin (*see* Table 1).

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