Detection of the Cell Proliferation Zone in Leaves by Using EdU

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[Abstract] Visualization of nuclei in S-phase cells in tissues is important for not only cell cycle research but also developmental research because morphogenesis is usually achieved by a combination of cell proliferation and cell expansion. Recently, DNA labeling with 5-ethynyl-2′-deoxyuridine (EdU), which is an analog of thymidine, has been used to visualize nuclei in S-phase cells to assess the activity of cell proliferation during development of plants. EdU is efficiently incorporated into newly synthesized DNA, and detection of EdU is based on the covalent reaction between EdU and Alexa Fluor® dye, which is one of useful fluorescent dyes; this allows us to use mild conditions for the assay without any DNA denaturation. This method could be easily applicable, and, indeed, has been used for various model and non-model plant species. Here, we have described a protocol developed for the detection of nuclei in S-phase cells in leaves.

Materials and Reagents

1. Plant tissues
2. Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Thermo Fisher Scientific, Molecular Probes™, catalog number: c10337)
3. 90 ml acetone (WAKO, catalog number: 019-00353)
4. 1x phosphate-buffered saline (PBS) (*e.g.* WAKO, catalog number: 314-90185)
5. 0.5% Triton X-100 in 1x PBS
6. Plastic tubes (PCR tube or 1.5-ml tube)
7. Aluminum foil
8. 90% acetone (see Recipes)
9. Fixative formalin-acetic acid-alcohol (FAA) (see Recipes)
10. 10 mM EdU stock solution (see Recipes)
11. 10 µM EdU solution (see Recipes)
12. Alexa Fluor® 488 azide solution (see Recipes)
13. Click-iT® EdU reaction buffer (see Recipes)
14. Click-iT® EdU buffer additive (see Recipes)

15. Reaction cocktail (see Recipes)

Equipment

1. Microscope (*e.g.* Nikon, model: ECLIPSE 80i)
2. Shaker
3. Vacuum pump
4. Desiccator
5. Razor
6. Tweezer

Procedure

1. For the experiment, select healthy plants and use seedlings, dissected leaves or leaf primordia, depending on the aim of the experiment. If you need to use older (mature) leaves, cut the leaves using a razor to obtain small-sized leaf discs (Figure 1A).
2. Incubate the tissues in PCR tubes filled with 100-200 µl 10 µM EdU solution for 2 to 3 h [Figure 1B; normally 2 h. Kotogány *et al.* (2010) showed that labeling with 10 µM EdU for 2 h is sufficient for slowly growing cultured cells]. Avoid soaking the tissues or seedlings thoroughly in the solution to ensure that DNA replication and cell division proceed with less stresses, if your plant material is not aquatic plant (Figure 1B).
3. After incubation, transfer the tissues gently to 1.5 ml tubes filled with iced 1 ml 90% acetone by tweezers and incubate on ice for 10 min.
4. Transfer the tissues gently to other 1.5 ml tubes by tweezers, and wash the tissues 3 times with 1 ml 1x PBS by using a pipette.
5. Remove the supernatant and fix the tissues with FAA for 2 to 3 h (the time for fixation depends on the plant materials or tissues). Decompression treatment using a pressure reducing pump for 15 min from the beginning or brief centrifugation are effective for the fixation.
6. Transfer the tissues gently to other 1.5 ml tubes by tweezers, and wash the tissues twice with 1 ml 0.5% Triton X-100 in 1x PBS by shaking (100-150 rpm) for 5 min.
7. After washing, prepare the reaction cocktail. (Do not prepare the cocktail before washing in order to avoid reducing the activity of the cocktail.)
8. Remove the supernatant and add the reaction cocktail to the tubes (generally, 50-100 µl/tube) and mix well by using a pipette.
9. Incubate the reaction cocktail for 30 min without any agitation and protect from light by wrapping the tubes with aluminum foil. To enhance the detection sensitivity, repeat step 8 with a fresh 50-100 µl/tube reaction cocktail.
10. After incubation, transfer the tissues gently to other 1.5 ml tubes by tweezers and wash the tissues 3 times with 1 ml 1x PBS by shaking (100-150 rpm) for 20 min;

protect from light by wrapping the tubes with aluminum foil.

11. EdU signals can be observed using a fluorescence microscope (Figure 1C; for the detection of EdU with Alexa Fluor® 488 azide, use 458-488-nm excitation with a green emission filter). EdU signals can be observed a few weeks or even months after the detection reaction if the tissues are stored in 1x PBS in dark, at 4 °C.

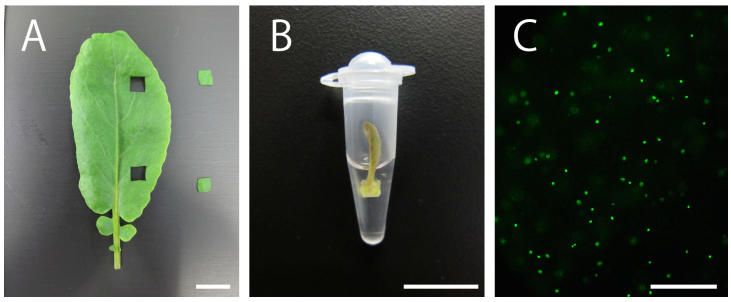

Figure 1. Plant material soaking in 10 μM EdU solution and visualization of nuclei in S-phase cells in the leaves of *Rorippa aquatica* (Brassicaceae). A. A leaf and leaf discs. B. A shoot apex subtending leaf primordia soaking in 10 μM EdU solution. C. Result of DNA labeling with EdU in leaves. This figure was taken under 20x objective. Bars = 1 cm in (A) and (B) and 100 μm in (C)

Representative data

For representative data, please see the papers of Ichihashi *et al.* (2014) and Nakayama *et al.* (2014).

Notes

1. Basically, perform all the steps at room temperature.
2. In all the steps, work gently to maintain the shape and structure of leaf primordia and leaves.
3. Nuclei in S-phase cells can be easily quantified by counting all the signals in a defined area.
4. This method is applicable for various plant tissues to detect nuclei in S-phase cells (*e.g.* shoot apical meristem).

Recipes

1. 90% acetone (100 ml)
   90 ml acetone
   10 ml H_2O
2. FAA (100 ml)

10 ml formaldehyde (37-40%)

5 ml glacial acetic acid

50 ml ethanol

35 ml H_2O

3. 10 mM EdU stock solution

   To prepare 10 mM EdU stock solution, add 2 ml of DMSO (Component C) to Component A in the kit and mix well

   Stored at -20 °C

   When stored as directed, this stock solution will remain stable for up to 1 year

4. 10 µM EdU solution

   To prepare 10 µM EdU solution, dilute 10 mM EdU stock solution to 10 µM by using H_2O

5. Alexa Fluor® 488 azide solution

   To prepare a working solution of Alexa Fluor® 488 azide, add 70 µl of DMSO to component B in the kit and mix well

   Stored at -20 °C

   When stored as directed, this solution will remain stable for up to 1 year

6. Click-iT® EdU reaction buffer

   To prepare Click-iT® EdU reaction buffer, add 3.6 ml of H_2O to 400 µl of Click-iT® EdU Reaction Buffer (Component D) in the kit and mix well

   Stored at 4 °C

   When stored as directed, this 1x buffer will remain stable for up to 6 months

7. Click-iT® EdU buffer additive

   To prepare Click-iT® EdU buffer additive, add 1 ml of H_2O to 200 mg of Click-iT® EdU Reaction Buffer additive (Component F) in the kit and mix well

   Stored at -20 °C

   When stored as directed, this buffer additive will remain stable for up to 1 year

8. Reaction cocktail

   It is important to add the ingredients in the order listed in the table to ensure that the reaction proceeds at an optimal rate

   Use the reaction cocktail as soon as possible

Table 1. Reaction cocktail

Reaction components	Volume
Click-iT® EdU reaction buffer	227.5 µl
$CuSO_4$ (Component E)	10 µl
Alexa Fluor® 488 azide solution	0.6 µl
Click-iT® EdU buffer additive	12.5 µl
Total	Approx. 250 µl

Acknowledgements

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