

Technique Notes

**Detection of S Phase in multiple *Drosophila* tissues utilizing the EdU labeling technique.**

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Introduction

Examining cellular proliferation via fluorescent labeling is essential to the study of molecular genetics, specifically being able to visualize defects in the cell cycle. Techniques developed previously to examine cellular proliferation include tritiated thymidine visualized with autoradiography and 5-bromo-2'-deoxyuridine (BrdU) immunohistochemistry (Leif *et al.*, 2004). Over the course of the past few decades the BrdU immunohistochemistry labeling method has been standard for labeling of cells in S-phase (Gratzner, 1982; Dolbeare, 1995); however, a newly developed technique utilizing 5-ethynyl-2'-deoxyuridine (EdU) promises to revolutionize the ability not only to detect DNA synthesis in cells progressing through the cell cycle (Buck *et al.*, 2008; Warren *et al.*, 2009) but also to facilitate multiple labeling of the tissues (Figure 1) (Capella *et al.*, 2008).

The Click-iT EdU assay (Invitrogen, Carlsbad, CA, USA) utilizes EdU, a thymidine analogue which, like its predecessor BrdU, is easily incorporated during DNA synthesis. Unlike the required antibody detection in BrdU methods, EdU is detected chemically through a "Click" reaction with a fluorescent azide probe (Buck *et al.*, 2008). The Click reaction is based on a [3+2] Huisgen Copper (I)-catalyzed cycloaddition reaction (Figure 1) (Salic and Mitchison, 2008; Rostostev *et al.*, 2002). The small size of the azide molecule utilized in EdU labeling allows it to access the ethynyl group of the incorporated EdU with ease. This highlights one of the many advantages provided by EdU labeling. Typically with BrdU a harsh denaturation step using HCl is needed to open the DNA to provide the anti-BrdU antibody access; however, because of its small size, EdU does not require this harsh denaturation step. This not only maintains the integrity of the DNA structure but the tissue structure as well (Bock *et al.*, 2006). EdU labeling affords many advantages over the BrdU labeling method. These advantages include: a significant reduction in protocol time, more gentle cellular treatments, and increased sensitivity (Zeng *et al.*, 2010). EdU has already been shown to have the ability to label the same cells as its predecessor BrdU in multiple studies (Capella *et al.*, 2008; Zeng *et al.*, 2010).

Described in the following is an adapted method for utilizing EdU to label S phase cells simultaneously in multiple *Drosophila* tissues, including: neuroblasts, salivary glands, and wing discs. This adapted method also includes ways to incorporate this technique with other widely used methods including the ability to examine M phase indices and S phase indices.

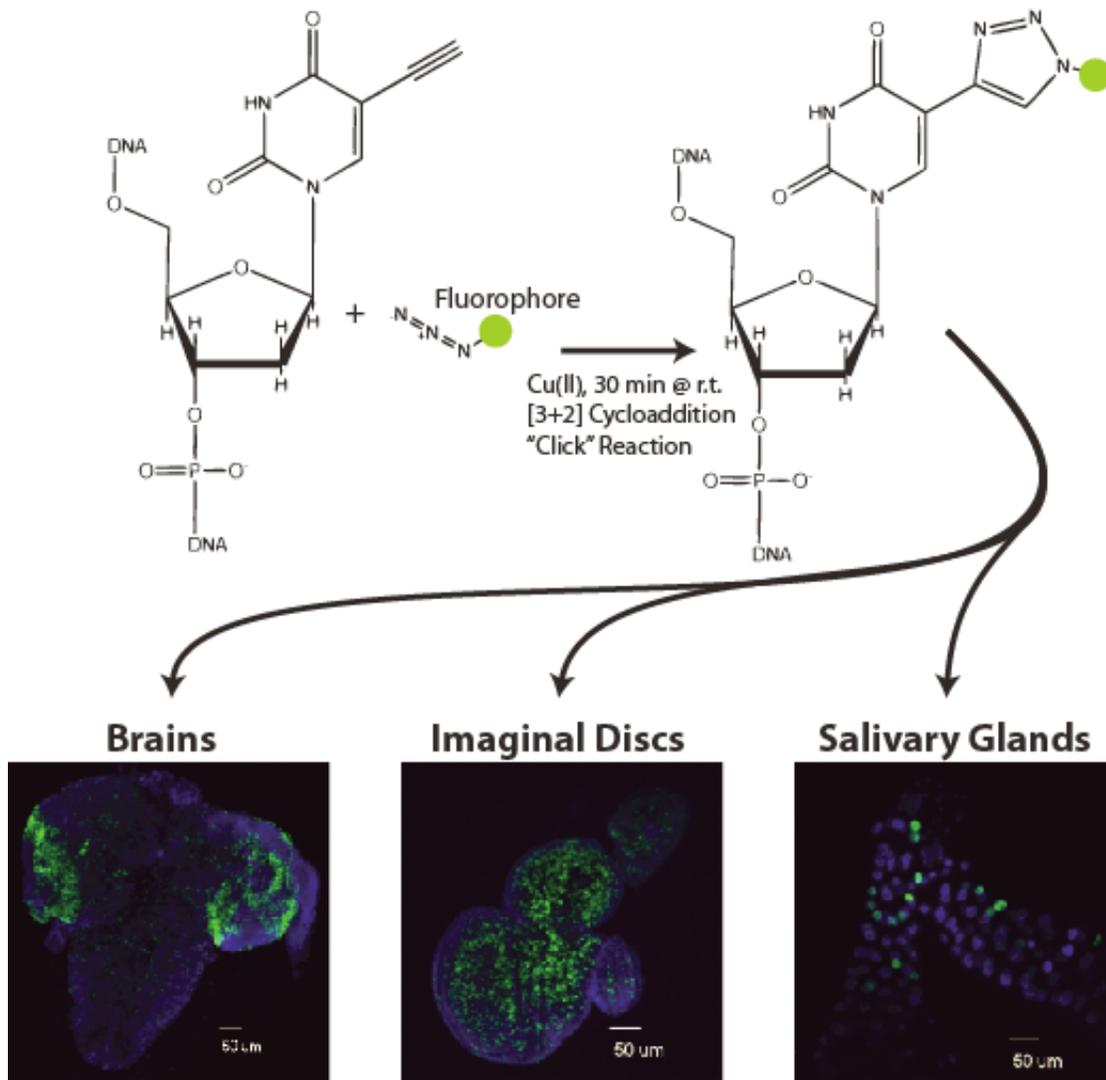


Figure 1. EdU, a thymidine analog is incorporated into DNA during synthesis in multiple *Drosophila* tissues. After incorporation of the EdU molecule a reaction utilizing CuSO_4 results in the 3+2 cycloaddition reaction depicted. The Alexa Fluor 488 fluorescent probe has been added and allows for the visualization of cells progressing through S phase before fixation. Below the reaction scheme various *Drosophila* tissues are shown where EdU has been incorporated (Green) and counterstained with for DNA (Blue).

Materials and Methods

Drosophila Stocks:

WT *Drosophila* stock was maintained at 25°C on *Drosophila* Diet Medium K12 (US Biological Cat # D9600-07B). The w^{1118} line was obtained from the Bloomington Stock Center (Flybase ID: FBst0006326).

Tissue Acquisition:

Wandering third in-star larva were selected and placed in a nine well plate containing 200 μL , HyQ[®] Grace's Unsupplemented Insect Cell Culture Medium (Hyclone, Logan, UT, USA). No.5 tweezers (Electron Microscopy Sciences, Hatfield, PA) were used to dissect and isolate the various tissues: brains, salivary glands, and wing imaginal discs. Upon dissection each tissue was isolated in a separate well containing 100 μl of fresh Grace's media divided for each treatment group.

EdU Labeling:

A 2 \times (30 μM) working solution was prepared in Grace's from the 10 mM EdU stock solution (Click-iT EdU Alexa Fluor Cell Proliferation Assay kit; Invitrogen, Carlsbad, CA, USA) and allowed to come to room temperature while isolating the desired tissue specimens. One half of the tissue was treated with Aphidicolin (Fischer Scientific, Pittsburgh, PA, USA) at 100 $\mu\text{g}/\text{ml}$ (diluted from a 1 mg/ml stock in DMSO) for 15 minutes and the other half treated with Grace's for the same 15 minute period. At the end of 15 minutes both solutions were removed from the wells housing the tissues, and the tissues were washed twice with 200 μL 3% Bovine Serum Albumin (BSA) (Fisher Scientific, Fair Lawn, NJ) in 1 \times PBS. After the wash was complete the BSA was removed and 100 μL of Fresh Grace's media was placed in each well followed by 100 μL of the 2 \times (30 μM) EdU solution (final EdU concentration 15 μM). Tissues were incubated for 30 minutes, and the EdU solution removed. 200 μL of 3% BSA in PBS was used to wash the tissues two times.

Tissue Fixation, Permeabilization and EdU Detection:

A 3.7% formaldehyde fixative was added for 5 minutes, removed, and the tissues were washed with 200 μL 3% BSA in PBS. The solution was removed and 200 μL of 0.1% Triton-X-100 was added in 1 \times PBS to each well for 20 minutes. The Click-It reaction cocktail was added containing 20 μL CuSO_4 , 430 μL of 1 \times Reaction Buffer, 50 μL 1 \times Buffer Additive, and 1.2 μL of the Alexa Fluor 4880Azide while the tissue was incubating in the permeabilization buffer. After removal of the permeabilization buffer, the tissues were washed with 200 μL of 3% BSA in PBS two times after which 200 μL of Click-iT reaction cocktail was added to each well for 30 minutes, removed, and again washed with 200 μL 3% BSA in PBS.

DNA Labeling:

To stain DNA a 1 \times (5 $\mu\text{g}/\text{mL}$) Hoescht33342 solution was added for 15 minutes. Each well was then washed with 1 \times PBS two times, and the tissues were transferred to glass slides and mounted using 7 μL of Vectashield (Vector Laboratories, Burlingame, CA) mounting medium per tissue specimen. A Lifterslip[™] (Thermo Fisher Scientific, Portsmouth, NH # 25X60i-2-4789) was placed on top of the prepared tissue. Slides were then placed at 4 $^{\circ}\text{C}$ until fluorescence could be visualized using an Olympus IX2-DSU Spinning Disc Confocal Microscope (Olympus America Inc., Center Valley, PA).

Brain Squashes:

For brain squashes, after incubation in the 15 μM 1 \times EdU solution, the brains were incubated for exactly 10 minutes in 0.5% Sodium Citrate. These brains were then lightly fixed with an 11:11:2 Acetic Acid, Methanol, and Water solution for 30 seconds. The brains were transferred to individual slides each containing a 5 μl dot of 1 \times PBS. A coverslip treated with SigmaCote[™] (Sigma Diagnostics. # SL2-25ML) was placed over the tissue, and a slide sandwich was created. The sandwich was prepared using a fresh slide, a piece of filter paper cut to the size of the slide placed in the middle, and the tissue specimen with the coverslip facing to the inside on the opposite side. The

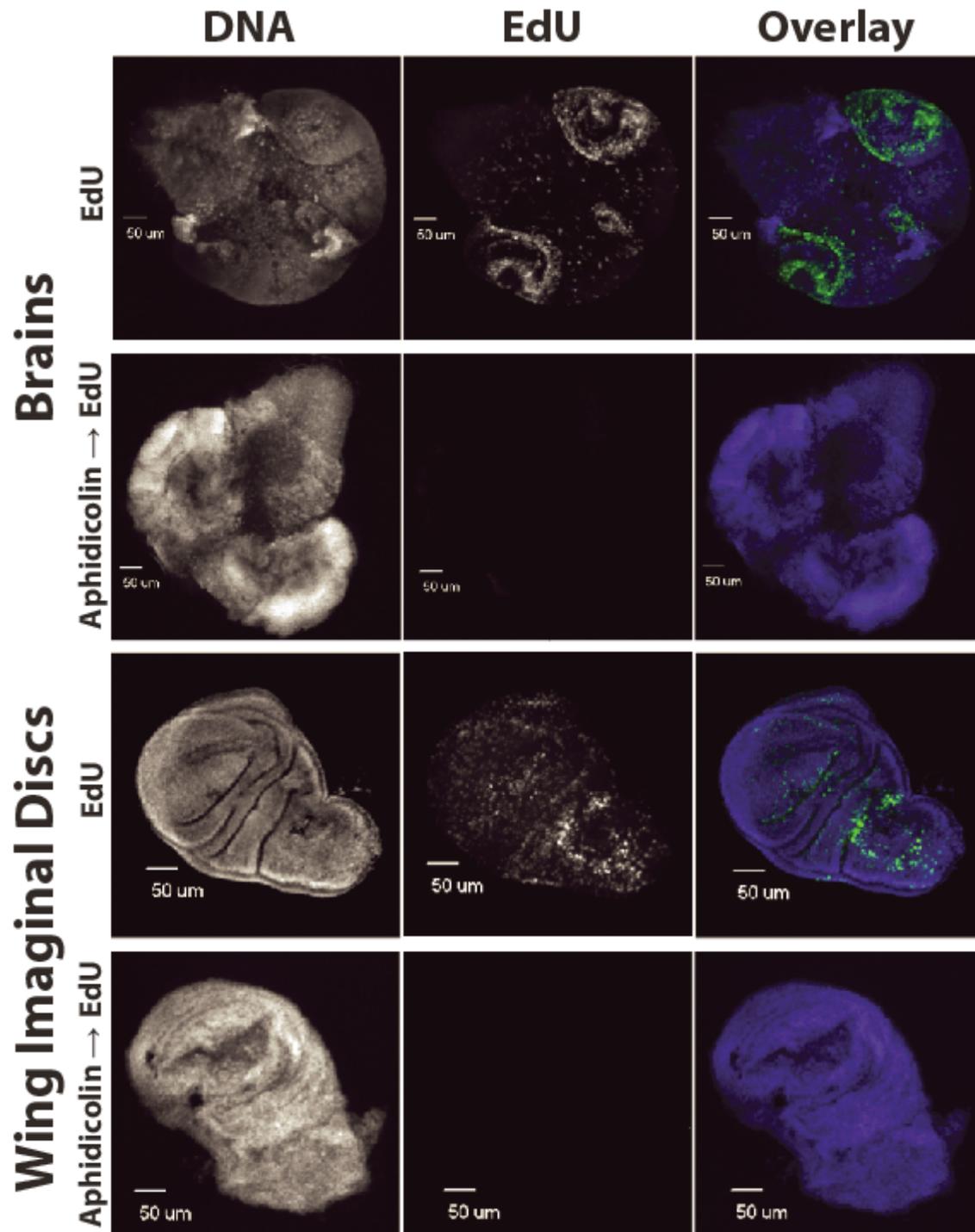


Figure 2. EdU and its detection are specific to cells undergoing DNA synthesis. Both *Drosophila* WT whole mount brain preps and Wing Imaginal discs were exposed to EdU with either a mock or pre-treatment with the DNA synthesis inhibitor Aphidicolin. DNA is stained with Hoescht 33342 and newly synthesized DNA that has incorporated EdU is visualized with a Alexa Fluor 488 probe. In all cases no appreciable detection of EdU is observed in tissue where DNA synthesis is blocked, whereas typical DNA replication patterns are observed in tissue where DNA replication is allowed to proceed.

slide sandwich was then placed in a toolmaker vise (Wilton, Cat #: 11715 Penn Tools, Maplewood, NJ, USA), and a digital torque wrench (Gearwrench #85071) was used to apply 15N of force to the slide sandwich for two minutes. After removing the slide from the vise, it was gently and slowly placed into a container of liquid nitrogen for approximately 5 seconds and removed. The coverslip was then popped off using a razor blade, and the steps were continued as described above in the subsequent sections, Tissue fixation/permeabilization and cellular DNA labeling.

Calculation of the M phase & S phase index were performed using 10 brain squash preparations, examining 10 fields of view per brain, counting the total number of cells present, the number of cells positive for EdU incorporation (S phase), and the number of mitotic figures (M phase).

Results

EdU labeling and detection are specific to cells undergoing DNA replication.

In order to evaluate the specificity of EdU labeling and detection, dissected tissues were pre-incubated with Aphidicolin, a potent inhibitor of DNA replication, and compared to mock treated tissues (Raff and Glover, 1988) (Figure 2). In all cases observed, in multiple tissue types, EdU incorporation and subsequent detection only occurred in those treatments where DNA replication was allowed to proceed normally. In wandering 3rd instar brains (Figure 2, top panel), typical DNA replication patterns were observed as characteristic optic lobe proliferation centers stain positive for EdU incorporation. EdU incorporation in wing imaginal discs is also consistent with previous BrdU studies as incorporation occurs in disperse cells due to the fact that cells in this tissue undergo asynchronous cell cycles (Phillips and Whittle, 1993).

EdU treatments may be used to generate S phase indices alongside M phase indices.

The harsh acid treatment of squashed larval brains required for BrdU detection compromises the integrity of the tissue. As a result the reliable detection of mitotic chromosomes is hampered. Due to the fact that EdU detection is much less harsh, it is more feasible to simultaneously quantitate mitotic and S phase indices (Figure 3, top panel). Figure 3 (bottom panel) illustrates typical fields of view from brain squash preparations. Multiple fields of view from multiple brains squashes were used to quantify the fraction of cells in either M Phase (#mitotic figures/total # of nuclei) and the fraction of cells in S phase (#cells positive for EdU/total # of nuclei). For wild-type *Drosophila* under the condition tested, S phase indices are $9.77 \times 10^{-2} \pm 1.9 \times 10^{-2}$ and M phase indices are $9.95 \times 10^{-4} \pm 2.70 \times 10^{-4}$.

Discussion

In summary, utilizing the EdU labeling technique we have stained multiple tissues, all of which are routinely utilized when studying the cell cycle and DNA replication in *Drosophila*. Not only does this technique afford shorter incubation times and the preservation of the cellular structure, but it also is all done *in vitro* without pulse feeding, as many of the BrdU assays employ. This new EdU assay is highly reproducible and cost effective compared to earlier techniques. Moreover, this technique is easily adapted to other research models where dissected tissues can be maintained for periods of time in culture media.

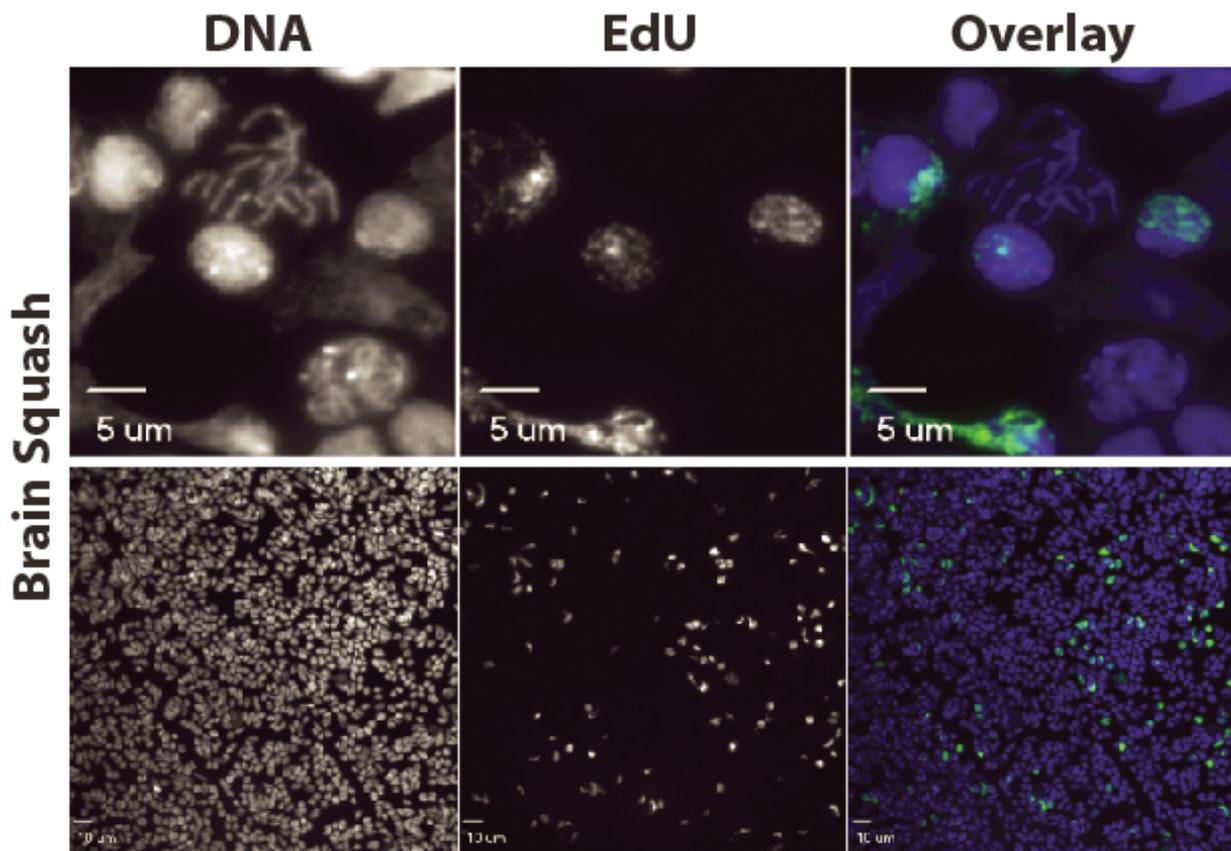


Figure 3. Examination of mitotic chromosomes and the quantitation of mitotic and S phase indices. Wandering 3rd instar larval brains were prepared as described and mitotic and S phase indices determined. Top panel demonstrates the ability of the method to maintain chromosome structure, and the bottom panel shows a typical field of view used for measuring M and S phase indices.

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References: Bock, V.D., H. Hiemstra, and J.H. van Maarsveen 2006, *European J. Org. Chem.* 1: 51-68; Buck, S.B., J. Bradford, K.R. Gee, B.J. Agnew, S.T. Clarke, and A. Salic 2008, *Biotechniques* 44: 927-929; Capella, P., F. Gasparri, M. Pulici, and J. Moll 2008, *Cytometry A* 73: 626-636; Dolbeare, F., 1995, *Histochem. J.* 27: 339-369; Gratzner, H.G., 1982, *Science* 218: 474-475; Leif, R.C., J.H. Stein, and R.M. Zucker 2004, *Cytometry A* 58: 45-52; Phillips, R.G., and J.R.S Whittle 1993, *Development* 118: 427-438; Raff, J.W., and D.M. Glover 1988, *J. Cell. Bio.* 107: 2009-2019; Rostovstev, V.V., G.L. Green, V.V. Fokin, and K.B. Sharpless 2002, *Angew Chem. Int. Ed. Engl.* 41: 2596-2599; Salic, A., and T.J. Mitchison 2008, *Proc. Natl. Acad. Sci. USA* 105: 2415-2420; Warren, M., K. Puskarczyk, and S.C. Chapman 2009, *Dev. Dyn.* 238: 944-949; Zeng, C., F. Pan, L.A. Jones, M.M. Lim, E.A. Griffin, Y.I. Sheline, M.A. Mintun, D.M. Holtzman, and R.H. Mach 2010, *Brain. Res.* 1319: 21-32.

Protocol

Reagents:

HyQ[®] Grace's Unsupplemented Insect Cell Culture Medium (Cat No. 30610.01, Hyclone, Logan, UT)

Click-It[™] EdU AlexaFluor[®] 488 Kit (Cat No. 10337, Invitrogen, Carlsbad, CA)

Bovine Serum Albumin (Cat. No. 9048-46-8, Fisher Scientific, Fair Lawn, NJ)

LIFTERS LIP Coverslips 25×60mm (25×60I-2-4789, Thermo Fisher Scientific, Portsmouth, NH)

Vectashield[®] Mounting Medium (Cat. No. H-1000, Vector Laboratories, Burlingame, CA)

Sigmacote[®] (Sigma Aldrich[™] Inc., St. Louis, MO)

Procedure:

EdU LABELING

1. Prepare a 2× (30 μM) EdU solution from the 10 mM EdU solution prepared from the Invitrogen kit in Grace's Cell Culture Medium (room temperature) and set aside.
 - * Add 1.5 μL of 10 mM EdU to 498.5 μL of Grace's – scale down if you are only doing a couple of specimens.
2. Dissect out desired *Drosophila* tissue in Grace's in a 9 well plate or 2 well depression slide.
3. Transfer tissue using No. 5 tweezers to a holding well with 100 μL of Grace's

****Helpful Hint:*** Judge the amount of Grace's you need based on the size of the well and the amount of tissue you are planning to stain, making sure to keep the appropriate concentration.

4. After obtaining all of the tissue samples desired add an equal volume of the 2× EdU solution to the well containing the tissue, resulting in a 1× (15 μM) EdU solution and incubate for 35 minutes.
 - i.e.*, 100 μL EdU to 100 μL Grace's and add brain
5. Pipette off the EdU solution

****ATTENTION if you plan to perform the brain squash proceed to step 21 ****

6. Rinse two times with 3% BSA in 1× PBS

****Helpful Hint:*** Limit the amount of time from the start of the dissection of the tissues to the incubation with EdU to the tissues that can be dissected in 40 minutes.

FIXATION/PERMEABILIZATION

7. Add a 3.7% Formaldehyde fixative for 10 minutes
8. Pipette off Formaldehyde fixative
9. Wash two times with 3% BSA
10. Add a 0.5% Triton-X in 1× PBS for 20 minutes

11. While incubating in step 10 prepare the Click-It Reaction Cocktail recipe listed under recipes
12. Pipette off Triton-X solution
13. Wash two times with 3% BSA in PBS

REACTION COCKTAIL

14. Add 200 μ l of the reaction cocktail per well and incubate for 30 minutes in the dark

ATTENTION: Protect the tissue from light throughout the remainder of the protocol.

15. Remove cocktail and wash two times with 3% BSA

ATTENTION: At this point if you have another staining protocol you wish to perform then continue to that protocol. If not continue to step 16.

16. Add a 1 \times Hoescht 33342 (5 μ g/mL) solution in 1 \times PBS and incubate for 20 minutes in the dark.
17. Wash each well two times with 1 \times PBS
18. Pipette approximately 5 μ l of PBS on to a new clean microscope slide and transfer the tissue from the well to the center of the PBS on the microscope slide.

Helpful Hint: The PBS will prevent the whole mount specimens from drying out while transferring multiple specimens to the same slide.

19. After transferring the tissue specimens, pipette off excess liquid and add approximately 10 μ l of Vectashield or other mounting media to the slide
20. Place a Liftersliptm coverslip on top and seal around the edges with nail polish.

BRAIN SQUASH

21. Remove the wash solution and add 200 μ l of 0.5% Sodium Citrate Solution
22. Remove Sodium Citrate Solution and add 200 μ l of 11:11:2 Acetic Acid, Methanol and Water to the well for 30 seconds.
23. While incubating pipette approximately 5 μ l of 1 \times PBS on to a slide
24. Remove the 11:11:2 fixative and wash with 1 \times PBS
25. Transfer each brain to a separate slide and add a Sigmacote[®] coverslip
26. Make a slide “sandwich” using a piece of paper cut to the size of the slide and another clean slide
27. Place the “sandwich” into the vise (and use a torque wrench to apply 15.0 N of force to the slide.
28. Remove the slide sandwich from the vise and remove the slide with the tissue specimen.
29. Carefully lower the slide into liquid nitrogen using forceps for approximately 5 seconds
30. Pull the slide out and use a razor blade to pop off the coverslip.

**Helpful Hint: To ensure the proper placement of the solution on to the tissue specimen we use a blue sharpie to draw a circle on the underside of the slide that circumnavigates the area the specimen occupies. It allows one to easily visualize the specimen when working with multiple.*

ATTENTION: Proceed back to step 9 and continue through to the end of the protocol with the only change being that the solutions will be pipetted directly on to the tissue specimen located on the slide.

RECIPES

0.5% Sodium Citrate (100 ml)

Sodium Citrate Dihydrate 0.5g final concentration

Add distilled H₂O to bring volume to 100 ml

11:11:2 Acetic Acid, Methanol, and Water (100 ml)

Acetic Acid 11 ml

Methanol 11 ml

Water 2 ml

10X Phosphate Buffered Saline (1L)

NaCl 80 g 1.37 Molar

KCl 2.0 g 26.8 mM

Na₂HPO₄ 14.4 g 101 mM

KH₂PO₄ 2.4 g 17.6 mM

Dissolve the above ingredients in 800mL of distilled H₂O and adjust the pH to 7.4. Adjust the volume to 1L and autoclave to sterilize.

30 μM EdU Solution

5-ethynyl-2'-deoxyuridine(EdU) 1.5 μL

Cell Culture Media 498.5 μL

Adjust amount of solution based on the amount of tissue to be stained.

Click-It Reaction Cocktail (500 μL) (All components are a part of the Invitrogen kit C10337)

1× Click-It Reaction Buffer 430 μL

CuSO₄ 20 μL

Alexa Fluor Azide 1.2 μL

Reaction Buffer Additive 50 μL

1× Click-It[®] Reaction Buffer is prepared by using 43 μL in 387 μL of distilled H₂O.

Reaction Buffer Additive is prepared by using 5 μL of buffer additive in 45 μL of H₂O.

Note: Instead of diluting the reaction buffer as described in the kit we make a working solution from the stock for each set of staining.

EQUIPMENT

Dissecting Scope equipped with oblique illumination setting.

Fluorescent capable microscope equipped with DAPI filter and GFP or other capable fluorescent filter dependent upon staining.

Wilton Toolmaker Vise, Cat #: 11715 Penn Tools, Maplewood, NJ, USA.

Torque Wrench fitted with a hex nut attachment with a visible readout that can apply 15N of force.



Using scanning electron microscope for documenting *Drosophilid* sperm head morphology.

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Introduction

Morphological features of sperm heads can be used to hypothesize evolutionary relationships among species (Talarico *et al.*, 2008; Meisner *et al.*, 2005; Jamieson *et al.*, 1999). We expect to find variability in drosophilid sperm head morphology, because there is already known variability in the sperm length. For example, *D. bifurca* has the longest sperm, approximately 6 cm (Pitnick *et al.*, 1995; Joly *et al.*, 1995). In addition the external and internal genitalic structures themselves vary and have traditionally been used for taxonomic and phylogenetic purposes (Patterson and Stone, 1952).

Drosophilid sperm have been investigated using bright field, fluorescence, and/or confocal microscopy (Desai *et al.*, 2009; Snook *et al.*, 2000; Joly *et al.*, 1991; Shoup 1967). The mature sperm head has been reported as long and cylindrical, similar to a needle (Tokuyasu, 1974). The mature sperm head is approximately 0.5 - 0.3 microns, and the tail one fourth of this width (Shoup, 1967). These dimensions are actually the limits of bright field microscopy; therefore, it lacks the ability to resolve and identify fine structural differences in the surface morphology of sperm heads among species.

Transmission electron microscopy (TEM) has yielded images demonstrating difference in sperm maturation and cellular structure (Shoup, 1967). Sperm head surface structure can only be indirectly inferred using the TEM and would be better understood using the scanning electron microscope (SEM), which provides the required resolution along with surface imaging capabilities. The SEM has been used to image the large sperm balls of *D. bifurca*; however, the sperm head is not visible these images (Bjork *et al.*, 2007; Bjork and Pitnick, 2006). This paper is the first to report on efforts to employ scanning electron microscopy (SEM) to document the morphology of drosophilid sperm heads.

Methods

The scanning electron microscope (SEM) sample preparation procedures are based upon the methods of Meisner *et al.* (2005), Michalik and Huber (2006), and Bjork and Pitnick (2006). Mature