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Adapted from the Click-iT EdU Imaging Kit protocol (C10340; Manual)

Clearing adapted from DOI: 10.1126/sciadv.aba0365

Table of Contents

Setup

Prepare Stock Solutions

PBS-T (1%)

Reagent	Quantity (for 500 mL)	Final concentration
PBS 1X	495mL	-
Tween-20	5mL	1%

4% BSA in PBS

- Dissolve 2g of BSA in 50mL of PBS.

F-ara-EdU (20mM stock in DMSO)

- F-ara-EdU (2′S)-2′-Deoxy-2′-fluoro-5-ethynyluridine, (Sigma #T511293; FW 270.22 g/mol)
    - *Note: technically, given the formula weight, this is an 18.5mM stock...*
    - Add 1 ml of DMSO or PBS to vial containing 5 mg F-ara-EdU to make a 20 mM stock solution.
    - After being dissolved, remaining F-ara-EdU stock solution is stable for up to 1 year when stored at –20 °C.
    - F-ara-EdU solution should be portioned into single use aliquots to avoid freeze thawing.
    - CAUTION: EdU is incorporated into DNA and is a potential mutagen. Proper protective clothing should be used when handling EdU. Waste, including stock solutions, used substrate, and water containing F-ara-EdU should be considered as hazardous.

Click-It stocks

Alexa Fluor 647 azide (Component B)

1. Prepare a master stock solution of the Alexa Fluor® azide (Component B): Add 70 µL of DMSO (Component C) to Component B, then mix well.

- After use, store any remaining working solution at ≤–20°C. When stored as directed, this working solution is stable for up to 1 year.

2. Prepare a *Dye Working Stock* by diluting the master stock 1:100 in DMSO (assuming that the final concentration for the master stock from the kit is 5uM)

Click-iT reaction buffer (Component D)

- Prepare a working solution of 1X Click-iT® EdU reaction buffer (Component D):
    - Transfer the solution (4 mL) in the Component D bottle to 36 mL of deionized water.
    - To make smaller amounts of 1X Click-iT® EdU reaction buffer, dilute volumes from the Component D bottle 1:10 with deionized water. After use, store any remaining 1X solution at 2–8˚C. When stored as directed, this 1X solution is stable for 6 months.

Click-iT buffer additive (Component F)

- To make a 10X stock solution of the Click-iT® EdU buffer additive (Component F): Add
    - 2 mL deionized water to the vial, then mix until fully dissolved.
- After use, store any remaining stock solution at ≤–20˚C.
- When stored as directed, this stock solution is stable for up to 1 year.
- If the solution develops a brown color, it has degraded and should be discarded.

Custom Click stocks

Azide Dye Stock

- picolyl-Azide-Dye: 4 mM in H2O (3 mg in 1 ml H2O) (final 8 µM)

Copper Sulfate Stock (CuSO$_4$.5H$_2$O; MW 249.68)

- Dissolve 250 mg CuSO$_4$.5H$_2$O in 5 ml H$_2$O for 200mM working stock
- Final concentration in reaction: 2mM

Sodium Ascorbate Working Stock (Buffer additive, NaC$_6$H$_7$O$_6$; MW 198.11)

- **NOTE: Make Fresh for each experiment**
- Dissolve 0.198 g in 1 mL H_2O for 100mM working stock
- Final concentration in reaction: 10 mM

Clearing stocks

Detergent Mix (1 L)

Reagent	Quantity (for 500 mL)	Final concentration
10% SDS	100mL	1%

- 1% SDS: 100 mL 10% SDS (may be filtered)
- 0.5% Deoxycholate: 5g Deoxycholate (wear a mask when handling the powder) 50 mM Tris-HCl (pH 7.5): 50 mL 1 M Tris-HCl (pH 8.0) 1 mM EDTA (pH 8.0): 2 mL 0.5 M EDTA (pH 8.0) 150 mM NaCl: 30 mL 5 M NaCl

Clearing Solution-1.1

- 8 to 10% (v/v) THEED (Sigma-Aldrich, 87600-100ML)
- 5% (v/v) Triton X-100 (Roth, 3051.2), and
- 5% (w/v) urea (Roth, X999.2) in dH2O

Materials

- 1.5 ml Microcentrifuge tubes (clear)
- Rocker
- Staining vials
- Click-iT EdU Alexa Fluor 647 Imaging Kit (ThermoFisher #C10340)

Protocol

1. F-ara-EdU Pulse Labeling

1. Collect embryos at the desired stage.
2. Dechorionate embryos in Filtered Natural Sea Water or ASW.
    - TODO: Test whether dechorionation is necessary. If not, will permit more pulse and longer chases throughout development.
3. Transfer embryos or larvae into 1.5 ml microcentrifuge tube or multi-well plate (e.g. 24-well plate) depending on desired pulse length.
    - If using a plate, you should pre-coat with 0.2% agarose to prevent embryos from sticking and allow for chorion expansion.

4. To optimize labeling efficiency and minimize toxicity, you should set up a test matrix of different concentrations and durations of F-ara-EdU labeling.
    - Include cultures treated with vehicle alone for controls.

	30 min	60 min	120 min	24 h
1µM				
10µM				
100µM				
0µM (no EdU control)		x	x	x

5. Add appropriate volume of F-ara-EdU (20mM stock) to achieve desired final concentration.
    - For example, to achieve a final concentration of 10µM, add 1µl of 20mM F-ara-EdU stock solution to 2ml of FNSW.
6. Incubate embryos in F-ara-EdU solution at 18°C for desired time interval.
7. If chasing, change back into FNSF + 1% Pen/Strep after pulse for duration of chase interval.
    - Transfer to a clean plate or tube to avoid carryover of f-ara-EdU (e.g. in agarose).
8. Proceed immediately to fixation and permeabilization (below).

2. Fixation & Permeabilization

1. Fix embryos overnight in 4% PFA in FNSW at 4°C in round bottom 2mL tubes.
    - Alternatively, you ***may*** fix embroys for ~1h at RT in 4% PFA in FNSW but O/N is better.
    - Can be left for up to 4 days?
2. After fixation, remove fixation buffer and wash embryos in 1mL PBS-T (1%) on rocker.

- 1x 5 min
- 2x 10 min

3. Proceed to next step.

3. Clearing (optional)

1. Remove PBS-T and add 1mL of prechilled acetone (-20°C) to each tube.
2. Keep at -20°C for a minimum of 2h.
3. Wash 3x with 1mL PBS-T (1%) for 5-10 min each.
4. OPTIONAL (not recommended in *conjunction* with detergent below) Add 500µl of proteinase K (75 µg/mL; Merck 1245680100) and rock for 5 min at RT.
    - Quench with 2x exchanges of 1mL glycine (2mg/mL)
5. **PREFERRED Wash 3x with 1mL Detergent mix for 20-30 min each at RT.**
6. Wash 3x with 1mL PBS-T (1%) for 5-10 min each.
7. Transfer embryos to glass vial containing 10mL of Clearing Solution-1.1
    - Gently rock for 3-6 hours at 37°C
8. Transfer back to 2mL round bottom tubes and wash 3x with 1mL PBS-T (1%) for 5-10 min each.

4. Blocking

1. Block embryos in 1mL 4% BSA in PBS for 1h at RT on rocker.
2. Wash embryos in 1mL 4% BSA in PBS 1 x 5 min on rocker.
3. Proceed to next step.

PAUSE POINT: Store at 4°C

5. Click-iT F-ara-EdU Fluorescent Labeling and Detection

1. Allow Click-iT kit components to come to room temperature before opening.
2. Prepare ***1X Click-iT® EdU buffer additive*** (Component F) by diluting the 10X stock solution 1:10 in deionized water. Prepare this solution fresh and use the solution on the same day.
3. Prepare ***Click-iT® reaction cocktail*** according to Table. It is important to add the ingredients in the order listed in the table; otherwise, the reaction will not proceed optimally. Use the Click-iT® reaction cocktail within 15 minutes of preparation.

- For each tube mix together:
  - ***430 µl of 1x Click-iT reaction buffer***
  - ***20 µl CuSO4*** (Component E)
  - ***1.2 µl fluorescent azide*** (Component B)
  - ***50 µl 1x reaction buffer additive*** (Component F; diluted above from 10x stock).
- Scale up the mixture for the number of samples to be treated and add 500 µl to each tube.
- It is important to use the cocktail within 15 min of preparation.
- It is good practice to include a control sample of cells not exposed to EdU. In addition, these cells are needed for single staining compensation controls for intracellular antigens or antigens stained with RPE, PE-tandem, or Qdot antibody conjugates.

Table 3. Click-iT® reaction cocktails.

Reaction components*	Number of coverslips						
	1	2	4	5	10	25	50
1X Click-iT® reaction buffer (prepared in step 1.4)	430 µL	860 µL	1.8 mL	2.2 mL	4.3 mL	10.7 mL	21.4 mL
$CuSO_4$ (Component E)	20 µL	40 µL	80 µL	100 µL	200 µL	500 µL	1 mL
Alexa Fluor® azide (prepared in step 1.3)	1.2 µL	2.5 µL	5 µL	6 µL	12.5 µL	31 µL	62 µL
Reaction buffer additive (prepared in step 4.1)	50 µL	100 µL	200 µL	250 µL	500 µL	1.25 mL	2.5 mL
Total volume	500 µL	1 mL	2 mL	2.5 mL	5 mL	12.5 mL	25 mL
***Note**: Add the ingredients in the order listed in the table.							

5. ALTERNATE Custom Click Rxn Table

Reaction Components / Reaction	**1**	**2**	**4**	**5**	**10**	**25**	**50**
1x PBS	394 µl	x µl	x µl	x ml	x ml	x ml	x ml
CuSO4 (200 mM)	5 µl	x µl	x µl	x µl	x µl	x µl	x µl
Fluorescent azide (4 mM)	1 µl	x µl	x µl	x µl	x µl	x µl	x µl

Reaction Components / Reaction	1	2	4	5	10	25	50
DMSO	50 µl	x µl	x µl	x µl	x µl	x µl	x ml
Sodium Ascorbate (100 mM; Fresh)	50 µl	x µl	x µl	x µl	x µl	x µl	x ml
Total volume	500 µl	x µl	x ml	x ml	x ml	x ml	x ml

* Add reagents in the order listed above. Use within 15 min of preparation.

* DMSO protects the DNA from free radicals generated by the Cu(I) catalyst.

* For lower dye concentrations, you can try 10 µl of 10µM dye in 500 µl reaction for a final conc. of 200nM.

1. Remove the blocking buffer.
2. Quick wash each sample *twice* with 1 mL of PBS. Remove the wash solution.
3. Add 0.5 mL of Click reaction cocktail to each tube. place on rocker.
4. Incubate for 30-60 minutes at room temperature, protected from light (longer gets deeper in hatchlings).
5. Remove the reaction cocktail and wash twice with 1 mL of 4% BSA in PBS.
6. Optional: Keep overnight in 4% BSA in PBS at 4°C to allow excess dye to diffuse out of yolk.

6. Staining DNA

1. Add 1µl of 1000X DAPI (**TODO: Need concentration**) to each tube.
2. Stain for 30-60 min at RT on rocker. (longer gets deeper into hatchlings).
3. Remove DAPI and wash once with 1 mL of PBS w/ 4% BSA.
4. Final wash in 1x PBS
5. Store at 4°C until imaging.

7. Mounting and Imaging

1. Mount on Matex in 1% agarose in Fluoromount-G
2. Image on confocal microscope.

Notes/Ideas for Future Tweaking:

- Background in yolk significantly reduced with pre-block with BSA.
    - May also try casein (milk) for blocking too. or LMW-Dextran.
- A few suggestions for optimizing:
- Can we reduce the amount of fluorescent dye?
- Longer washes after Click (trying ON soak in PBS currently)
- Wash in high DMSO (≤50% w/v) to remove unbound dye?
- 5% Urea wash to disrupt hydrophobic interactions with yolk?
- Can likely scale down the Click Rxn to 250µl in 2mL tubes. (This did not work so well first time.)
- Probably need to consider:
    - Increasing the dose >100µM for shorter pulses
        - Need to read up on what is usual dosing for EdU in other systems (e.g. zebrafish embryos; 400 µM EdU for 1 h at room temperature?)

    - Increasing the pulse time (1 hour is not really labeling much, and 24 hours seems good, but maybe because some cells are cycling twice?)
    -

Experiments

F-ara-EdU short chase

- Pulse St20 embryos for 1 hr with 100µM F-ara-Edu
    - 5mL FNSW required per dish
        - 25µl 20mM F-ara-EdU per 5mL FNSW
- change back into FNSF + 1% Pen/Strep after pulse
    - Reserve some 0hr chase embryos for comparison
- Collect after:
    - 0h
    - 1h
    - 2h
    - 4h
    - 8h
    - 12h
    - 24h

    Results

    - Need to wash embryos more thoroughly after Click Rxn
    - May need higher dose (400µM?) and/or longer pulse for better labeling

± chorion

Question: Does the chorion affect EdU incorporation?

- Pulsed St26 embryos ± chorion in 50µM F-ara-Edu (12.5µl per 5ml FNSF) for:
    - 1h
    - 24h
- Blocked for 1h
- Only used 250µl Click Rxn
- Result for 1 hr is very weak/low staining with moderately high background:
    - likely because of less proliferation at later stage, short pulse, and/or accessibility
- Need to assess the 24 hr pulse as well
    - 24 hour pulse click Rxn performed in 500 mL reaction
    - 24 hour pulse much stronger and cleaner

Results

- Lower levels of F-ara-EdU incorporation in embryos with chorion
- Interesting structures observed on anterior side.

Suggestions from the Lab

- Bring dye conentration down to ~50nM (vs 5µM final in standard ClickIt kit)
    - Azdye 647 - azide plus (Click chemistry tools)
    - 50nM
    - On ice, one minute
    - Get Richard's protocol for EdU-click
        - 1 mM CuSO4
        - 200 nM AzPlus-dye in PBS-T w/ 10% DMSO (This is 2 orders of magnitude lower than the Alexa647 in the ClickIt kit)
        - 5 mM sodium ascorbate
    - Stocks:
        - 100mM CuSO4
        - 100mM sodium Ascorbate