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1. F-ara-EdU Pulse Labeling of Doryteuthis pealeii Embryos and hatchlings with optional clearing

Clearing adapted from DOI: 10.1126/sciadv.aba0365

1.1. Table of Contents

1.2. Setup

1.2.1. Prepare Stock Solutions

1.2.1.1. PBS-T (1%)

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

1.2.1.2. 4% BSA in PBS

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

1.2.1.3. High salt PBS (for better DAPI penetration)

Reagent	Quantity (for 500 mL)	Final concentration
NaCl	1.06g	500 mM (363mM added to 137mM in std PBS)
PBS 1x	to 50 mL	

1.2.1.4. 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.

1.2.2. Click reaction stocks

1.2.2.1. Azide Dye Stock

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

1.2.2.2. 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

1.2.2.3. 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

1.2.3. Clearing stocks

1.2.3.1. Detergent Mix (1 L) *(from Jenny McCarthy)*

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

1.2.3.2. Clearing Solution-1.1

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

1.2.4. Materials

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

1.3. Protocol

1.3.1. F-ara-EdU Nucleotide Pulse Labeling

1. Collect embryos at the desired stage in FNSW.
2. Dechorionate embryos to enhance EdU penetration.
3. Transfer embryos or larvae into round bottom 2ml 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				

	30 min	60 min	120 min	24 h
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 FNSW + 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).
    - Be sure to thoroughly exchange the media to remove any residual F-ara-EdU.
8. Proceed immediately to fixation and permeabilization (below).

1.3.2. (Alternative) F-ara-EdU Nucleotide Injection into >hatchlings

1.3.3. 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 fixative and wash embryos in 1mL PBS-T (1%) on rocker.

- 3x 10 min

3. Proceed to next step.

1.3.4. Target Retrieval

- [OPTIONAL] Remove PBS-T and add 1mL of prechilled acetone (-20°C) to each tube.
    - Keep at -20°C for a minimum of 2h.
    - This can be helpful if there is excess melanin in the tissue, and also generally helps to reduce the time required for tissue clearing.
    - Generally not needed if you can afford to spend more time in clearing solution later.
    - Wash 3x with 1mL PBS-T (1%) for 5-10 min each.
- [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)

1. **[PREFERRED]** Wash 3x with 1mL Detergent mix for 20-30 min each at RT.
2. Wash 3x with 1mL PBS-T (1%) for 5-10 min each.

1.3.5. [OPTIONAL] Blocking

- D. pealeii embryos are excessively yolky. Blocking can help reduce non-specific binding of dye. It's not a perfect solution, and the dye will naturally leach out over 1-2 days anyway. But this may help a bit. Really only matters for earlier embryos with a lot of yolk.

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

PAUSE POINT: Store at 4°C

1.3.6. Click Fluorescent Labeling and Detection

1. Allow Pre-made stocks to come to room temperature before opening.
2. Prepare fresh Sodium Ascorbate Additive
3. Prepare ***Click reaction cocktail*** according to Table below.
    - 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.
    - The clock starts upon the addition of Sodium Ascorbate when the Cu(I) catalyst is generated.

- For each tube mix together:
    - ***4394 µl of 1x PBS or other buffer***
    - ***25 µl CuSO4 (200mM)***
    - ***1 µl Fluorescent azide (4 mM)***
    - ***50 µl DMSO***
    - ***50 µl Sodium Ascorbate (100 mM; Fresh)***
- Scale up the mixture for the number of samples to be treated and add 500 µl of the master mix 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.

1.3.6.1. Click Rxn Scaling 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
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 current 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 and 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 PBS-T.
6. Optional: Keep overnight (or longer) in PBS at 4°C to allow excess dye to diffuse out of yolk.

1.3.7. Staining DNA

1. Add 1µl of 1000X DAPI (**TODO: Need concentration**) to each tube.
    - For better/faster penetrance, change tube to high-salt PBS when staining.
2. Stain for minimum of 60 min at RT on rocker. (or O/N at 4°C; The longer you stain with high-salt DAPI, the deeper the penetration).
3. Remove DAPI and wash once with 1 mL of PBS-T.

1.3.8. Clearing (Optional)

1. Transfer embryos to glass vial containing 10mL of Clearing Solution-1.1
    - Gently rock for a minimum of 3-6 hours at 37°C
    - I have left them @37°C for >72 hours with little ill effect.
2. You *may* be able to store embryos in Clearing Solution-1.1, but refrigeration appears to make them a bit cloudier at this point.
    - You can also consider changing back to PBS for storage

1.3.9. Mounting and Imaging

1. Mount on Matex in 1% agarose in Vectashield Plus for RI matching (or Fluoromount-G but does not match RI as well).
2. Image on confocal microscope.

2. 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)
- 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?)