Updated: Jul 3, 2024

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

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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.

Alexa Fluor 647 azide (Component B)

1. Prepare a working 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.

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.

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 1.5ml 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. 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

4. 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.							

For 250µl reactions:

Reaction Components / Reaction	1	2	4	5	10	25	50
1x Click-iT reaction buffer	215 µl	430 µl	900 µl	1.1 ml	2.15 ml	5.35 ml	10.7 ml
CuSO4 (Component E)	10 µl	20 µl	40 µl	50 µl	100 µl	250 µl	500 µl
Fluorescent azide (Component B)	0.6 µl	1.25 µl	2.5 µl	3 µl	6.25 µl	15.5 µl	31 µl
1x reaction buffer additive (Component F)	25 µl	50 µl	100 µl	125 µl	250 µl	625 µl	1.25 ml

Reaction Components / Reaction	1	2	4	5	10	25	50
Total volume	250 µl	500 µl	1 ml	1.25 ml	2.5 ml	6.25 ml	12.5 ml

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-iT reaction cocktail to each tube. place on rocker.
4. Incubate for 30 minutes at room temperature, protected from light.
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.

5. Staining DNA

1. Add 1µl of 1000X DAPI (**TODO: Need concentration**) to each tube.
2. Stain for 10-30 min at RT on rocker.
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.

6. 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.
- 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.