Developed by Singh Lab

Step-by-Step Protocol: 5-EdU Incorporation and Detection *via* Click Chemistry in Zebrafish

Description:

This protocol provides a detailed guide to perform Copper (Cu(I))-catalyzed Azide-Alkyne Click chemistry reactions (CuAAC) in zebrafish post 5-EdU incorporation.

Required Reagents:

E3 Medium for zebrafish embryos
5-EdU (Cat. No. CLK-N001)
Cell Reaction Buffer Kit (BTTAA based) (Cat. No. CLK-073)
AZDye647-Picolyl-Azide (Cat. No. CLK-1300A)
Phosphate Buffered Saline (PBS) (pH 7.4)
Washing solution (3% BSA in PBS (PBSB))
Fixation solution (4% Paraformaldehyde (PFA) or Formaldehyde (FA) & 10% Triton-X)
Permeabilization solution (0.5% PBST (PBS + 0.5% Triton-X))
DAPI/Hoechst (optional)
Mounting medium for imaging

1. Introduction

A common approach for DNA synthesis monitoring relies on incorporation of ethynyl-labeled deoxyuridine (5-EdU) instead of their natural counterpart thymidine.[1,2,3,4,5] Labeling of the resulting ethynyl-functionalized DNA is achieved *via* Copper (Cu(I))-catalyzed Azide-Alkyne Click chemistry reactions (CuAAC) that allows to introduce a fluorescent group for subsequent microscopic imaging.

DNA synthesis monitoring with 5-EdU in zebrafish is performed in a four-step procedure:

1) Incorporation of 5-EdU into the DNA
2) Fixation and permeabilization
3) CLICK labeling of ethynyl-functionalized DNA via CuAAC reaction
4) Mounting and Imaging

A step-by-step protocol for 5-EdU incorporation followed by DNA synthesis detection *via* Copper (Cu(I))-catalyzed Azide-Alkyne Click chemistry reactions (CuAAC) in zebrafish is outlined below, however, individual optimization might be required for different CuAAC labeling experiments as well as for critical reaction parameter e.g. final CuSO4 concentration, $CuSO_4$:BTTAA ratio, detection reagent concentration.

2. Preparation of stock solutions

Adjustments might be required if different assay volumes or final compound concentrations are used.

2.1 CuAAC Cell Reaction Buffer Kit (BTTAA based)

The following ingredients listed below are supplied with the CuAAC Cell Reaction Buffer Kit (BTTAA based) (Cat. No. CLK-073).

2.1.1 BTTAA stock solution (Cu(I) stabilizing ligand)

- Add an appropriate amount of ddH_2O (Tab. 1), vortex until the compound is completely dissolved and spin down briefly.
- Prepare 105 µl aliquots and store at -20 °C.

Table 1 Volume of ddH_2O required for a 50 mM BTTAA stock solution.

BTTAA	Concentration of stock solution	Amount of ddH_2O
25 mg	50 mM	1163 µl

2.1.2 $CuSO_4$ stock solution (copper source)

- Add an appropriate amount of ddH_2O (Tab. 2), vortex until the compound is completely dissolved and spin down briefly.
- Prepare 25 µl aliquots and store at -20 °C.

Table 2 Volume of ddH_2O required for a 100 mM $CuSO_4$ stock solution.

$CuSO_4$	Concentration of stock solution	Amount of ddH_2O
10 mg	100 mM	628 µl

2.1.3 Na-Ascorbate stock solution (reduction reagent)

- Add an appropriate amount of ddH_2O (Tab. 3), vortex until the compound is completely dissolved and spin down briefly.
- Prepare 101 µl aliquots and store at -20 °C.

Table 3 Volume of ddH_2O required for a 1 M Na-Ascorbate stock solution.

Na-Ascorbate	Concentration of stock solution	Amount of ddH_2O
200 mg	1 M	1010 µl

Please note: Do not use solutions that appear brown. *Freshly prepared, fully functional Na-Ascorbate solutions are colorless to slightly yellow and turn brown upon oxidization thereby losing their reduction capability.*

2.2 AZDye647-Picolyl-Azide

- Add an appropriate amount of DMSO (Tab. 4), vortex until the compound is completely dissolved and spin down briefly.
- Prepare 100 µl aliquots and store at -20 °C.

Table 4 Volume of DMSO required for a 0.5 mM AZDye647-Picolyl-Azide stock solution.

AZDye647-Picolyl-Azide	Concentration of stock solution	Amount of DMSO
1 mg	200 mM	1885 µl

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3. 5-EdU Treatment of Zebrafish Larvae

3.1 E3 medium for zebrafish embryos

- To prepare a 60X stock solution, dissolve the ingredients in ddH_2O (Tab. 5), to a final volume of 2 l. Adjust the pH to 7.2 with NaOH. Autoclave. Store in a fridge.

Table 5 Preparation of 60x E3 medium.

Component	Amount	
NaCl	34.8 g	
KCl	1.6 g	
$CaCl_2$ (*2 H_2O)	5.8 g	
$MgCl_2$ (*2 H_2O)	9.78 g	

- To prepare 1X medium, dilute 16.5 ml of the 60X stock to 1 l (Tab. 6). Add 100 µL of 1% methylene blue. Store at room temperature.

Table 6 Concentrations of 1x E3 medium.

Component	Working concentration
NaCl	5 mM
KCl	0.17 mM
$CaCl_2$	0.33 mM
$MgCl_2$	0.33 M

3.2 5-EdU stock solution

- Add an appropriate amount of DMSO (Tab. 7), vortex until the compound is completely dissolved and spin down briefly.
- Prepare aliquots to avoid repeated freeze-thaw-cycles and store at -20 °C.
- The solution is stable up to 1 year at -20 °C

Table 7 Volume of DMSO required for a 200 mM 5-EdU stock solution.

5-EdU	Concentration of stock solution	Amount of DMSO
25 mg	200 mM	495 µl

- To prepare a 10 mM 5-EdU solution, add an appropriate amount of E3 media (Tab. 8) in a 1.5 ml tube:

Table 8 Volume of E3 medium required for a 10 mM 5-EdU working solution.

Σ	200 mM 5-EdU stock solution	Concentration of working solution	Amount of E3 medium
1	12.5 µl	10 mM	237.5 µl
4	50 µl	10 mM	950 µl

Please note: *The 5-EdU working solution contains 5 % DMSO which is tolerated by larvae for short duration of time.*

3.3 5-EdU incubation

- Place 5-7 10 dpf larvae (15 larvae at 3–5 dpf) in a 50 ml Falcon tube.
- Remove all medium using a glass pipette.
- Add 250 µL 5-EdU solution to the tube.
- Transfer the Falcon tube to ice for 4 minutes.
- Add 50 ml of 28°C E3 media (kept in an embryo incubator).
- Lay the Falcon tube on its side on a shaker to aid larvae recovery.
- Once most larvae have recovered, transfer the Falcon tube to a 28°C incubator.
- Chase period: 4 hours.

4. Fixation

- Transfer larvae to a 15 ml Falcon tube.
- Fix with 9.5 ml 4% PFA (or FA) + 1 ml of 10% Triton-X (final concentration: 1% Triton-X + 4% PFA).
- Shake at room temperature for 90 minutes.
- Wash twice with PBS.
- Transfer larvae to a Petri dish.
- Using fine forceps, remove tail, skin, eyes, and brain.
- Transfer dissected samples to a 2 ml tube with PBS.
- Remove PBS, add 0.5% PBST, and shake for 10 minutes.

Please note: *Dissection is only used for labelling of internal organs at 10 dpf. At 3 - 5 dpf, no dissection is needed.*

5. Preparation of CLICK reaction cocktail

Please note: Prepare CLICK reaction cocktail freshly for each experiment.

- Allow all solutions to warm up to room temperature.
- Refer to Tab. 9 for appropriate amounts of compound stock solutions.

Table 9 Pipetting scheme for CLICK reaction cocktail. Please add the compounds exactly in the order described below. Keep in dark.

Component	Stock Conc.	Final Conc.	Volumne for 450 µl pre-mix
$CuSO_4$	100 mM	2 mM	10 µl
BTTAA	50 mM	4 mM	40 µl
Na-Phosphate reaction buffer, pH 7	100 mM	-	380 µl
AZDye647-Picolyl-Azide	0.5 mM	20 µM	20 µl

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6. CLICK-labeling of fixated and permeabilized 5-EdU-labeled Zebrafish Larvae

- Transfer samples to a 0.5 ml tube using a glass pipette.
- Add 450 µL PBSB (3% BSA + PBS).
- Turn the tube 2-4 times, then remove the solution using a glass pipette.
- Repeat PBSB wash once more. Remove PBSB.
- Add 225 µL of CLICK reaction cocktail to each tube.
- Shake protected from light for 5 minutes.
- Add 25 µL 1M Sodium Ascorbate to each tube.
- Shake protected from light for 20 minutes.
- Remove CLICK reaction cocktail using a glass pipette.
- Mix 25 µL 1M Sodium Ascorbate with CLICK reaction cocktail, add 250 µL to each tube.
- Shake protected from light for 25 minutes.
- Wash 1-2 times with PBS (rapid washes).
- Transfer samples to a 2 ml tube with fresh PBS.
- Store at 4°C overnight.

7. Optional DAPI/Hoechst Staining

- Remove PBS and replace with 0.5% PBST + 1:1000 DAPI/Hoechst.
- Shake at RT for 60 minutes or overnight in a cold room.

8. Mounting and Imaging

- In a Petri dish with PBS, dissect out the liver.
- Mount the sample on a slide.
- Slides can be imaged within one week.

Notes and Precautions:

- Always protect CLICK reaction cocktail from light.
- Ensure proper shaking during recovery and incubation steps.
- Rapid washes in PBS are sufficient.
- Proper liver dissection is key for accurate imaging.

Selected References:

[1] Salic *et al.* (2008) A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc. Natl. Acad. Sci. USA* **105**:2415.
[2] Li *et al.* (2010) Fluorogenic "click" reaction for labeling and detection of DNA in proliferating cells. *Biotechniques* **49 (1)**:525.
[3] Diermeier-Daucher *et al.* (2009) Cell Type Specific Applicability of 5-Ethynyl-2'-deoxyuridine (EdU) for Dynamic Proliferation Assesment in Flow Cytometry. *Cytometry A* **75A**:535.
[4] Darzynkiewicz *et al.* (2011) Cytometry of DNA Replication and RNA synthesis: Historical Perspective and Recent Advances Based on "Click Chemistry". *Cytometry A* **79A**:328.
[5] Diermeier-Daucher *et al.* (2010) Dynamic Proliferation Assessment in Flow Cytometry. *Curr. Protoc. Cell Biol.* **48**:8.6.1.

Manuscript where this protocol was partially used:

Eski *et al.* (2025) Cholangiocytes contribute to hepatocyte regeneration after partial liver injury during growth spurt in zebrafish. *Nat Commun* **16**:5260.