Click-ExM enables expansion microscopy for all biomolecules

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Method Article

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Abstract

We describe the development of click-expansion microscopy (Click-ExM), which integrates click-labeling into ExM to enable a“one-stop-shop” method for nanoscale imaging of various types of biomolecules. Using 18 clickable labels for click-ExM imaging of DNA, RNA, proteins, lipids, glycans and small molecules, we demonstrate its universality, compatibility with signal-amplification techniques, and broad applications in cellular and tissue imaging. The click-labeling and ExM steps could be finished within two days. This step-by-step protocol is related to the publication “Click-ExM enables expansion microscopy for all biomolecules” in *Nature Methods*.

Introduction

By physically expanding proteins or RNA of fixed specimens embedded in a swellable polymer hydrogel, expansion microscopy (ExM) enables nanoscale imaging by using conventional diffraction-limited microscopes. As a critical step of ExM, the biomolecules or the labeled fluorophores need to be covalently anchored into the polymer network and preserved during homogenization of the fixed cells (e.g., by strong protease digestion) to ensure isotropic expansion. This imposes a great challenge to develop tailored protocols for specific imaging targets including proteins and RNA; however, other types of biomolecules such as lipids, glycans, and small molecules remain challenging for ExM.

To expand the applicability of ExM, we developed click-ExM, a variant of ExM, into which a unified method to label, anchor, and preserve fluorescent signals for all kinds of biomolecules is integrated. To develop such a unified protocol, we exploited click-labeling, which has emerged as a versatile tool for fluorescence imaging of various biomolecules. DNA, RNA, proteins, glycans, and lipids can all be metabolically labeled with a bioorthogonal or “clickable” functional group (e.g., azide or alkyne), which is subsequently conjugated with fluorescent probes via bioorthogonal chemistry such as Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC or click chemistry) and copper-free click chemistry. In click-ExM, the metabolically labeled biomolecules are reacted with azide-biotin or alkyne-biotin via click chemistry, followed by staining with fluorescently labeled streptavidin. The streptavidin-fluorophore conjugates serve as a tri-functional probe, which binds biotin, anchors into the gel, and presents fluorescence.

Reagents

All chemical reagents were obtained from commercial suppliers, and used without further purification. Copper (II) sulfate pentahydrate (C8027), (+)-sodium L-ascorbate (A4034), glutaraldehyde (G7651), acrylamide (A9099), N,N'-methylenebisacrylamide (M7279), sodium acrylate (408220), N,N,N',N'-tetramethylethylenediamine (TEMED; T7024), ammonium persulfate (APS, A3678), 2-[4-{(bis[(1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl]amino)methyl}-1H-1,2,3-triazol-1-yl]acetic acid (BTTAA; 1236) and azide-PEG_3-biotin (AZ104) were purchased from Click Chemistry Tools. Acryloyl-X (AcX; A20770), streptavidin Alexa

Fluor 488 (S32354) and streptavidin Alexa Fluor 555 (S32355) were purchased from Thermo Scientific. Saponin (482689) were purchased from J&K Scientific. Proteinase K (P8107S) were purchased from NEB.

Equipment

Fluorescent microscope (e.g., wide-field, confocal microscope)

Procedure

Click-ExM imaging of Cho-contained phospholipids

Metabolic incorporation of Alk-Cho

1. Prepare 100 mM Alk-Cho stock solution using ddH_2O. Then prepare the labeling medium by diluting stock solution into cell culture medium to a final concentration of 100-200 uM.

Note: Other lipid labels are usually dissolved in DMSO, and the labeling medium should be vortexed vigorously before being added to cell cultures.

2. Culture the cells in the labeling medium for 12 h at 37 ℃.

Cell fixation and permeabilization (at room temperature, r.t.)

3. Wash cells with PBS for three times. Fix cells with 3% (w/v) formaldehyde and 0.1% (v/v) glutaraldehyde in PBS for 15 min. Treat cells with 0.1% (w/v) sodium borohydride for 7 min, and wash three times with 100 mM glycine in PBS.

4. Permeabilize cells with 0.1% (w/v) saponin in PBS for 5 min and washed three times with PBS.

Pause point: If necessary, samples could be stored temporarily at 4 ℃, but long-time storage may cause potential signal loss or distortion.

Click labeling with azide-biotin (at r.t.)

5. Prepare 200 uL click reaction mixture as the follows.

188 uL 1×PBS

1 uL azide-PEG_3-biotin (10 mM)

1 uL BTTAA/$CuSO_4$ complex (6:1, mol/mol, 10 mM)

10 uL sodium ascorbate (50 mM, freshly prepared, the final step)

6. Incubate cells with the mixture for 1 h with gentle shaking.

7. Wash with PBS for five times.

8. Incubate cells with 5 ug mL^{-1} streptavidin-dye in PBS containing 1% (w/v) BSA for 1 h, and washed three times with PBS.

Pause point: If necessary, samples could be stored temporarily at 4 ℃, but long-time storage may cause potential signal loss or distortion.

Gelation, digestion and expansion

9. Incubate samples with 0.1 mg mL^{-1} AcX in PBS at r.t. overnight, or incubate the cells with 0.25% (v/v) GA in PBS for 10 min.

10. Wash samples three times with PBS.

11. Prepare monomer solution as the follows.

1×PBS

2 M NaCl

2.5% (w/v) acrylamide

0.15% (w/v) N,N'-methylenebisacrylamide

8.625% (w/v) sodium acrylate

12. Prepare 1 mL gelation solution as the follows.

940 uL monomer solution

20 uL ddH_2O

20 uL 10% (w/w) TEMED (freshly prepared)

20 uL 10% (w/w) APS (freshly prepared, the final step)

13. Incubate samples with the gelation solution at 4 °C for 5 min, and then transfer to a humidified 37 °C incubator for 1 h for gelation.

Note: Before placing the samples in a 37 °C incubator, keep the solutions chilled to 4°C, and avoid warming the gelling solution.

14. Prepare digestion solution as the follows.

50 mM Tris, pH 8.0

1 mM EDTA

0.1% (v/v) Triton X-100

0.8 M guanidine HCl

8 units mL^{-1} proteinase K (added before use)

15. Digest the hydrogel in digestion buffer at 37 °C for different time. In general, 4 h for AcX-anchored samples and 2 h for GA-anchored samples.

Pause point: If necessary, the gel could be stored in PBS temporarily at 4 ℃, but long-time storage may cause potential signal loss or distortion.

16. Place the hydrogel into ddH_2O to expand. Change water every 20 min until expansion was complete.

17. Optional: Stain the nuclei with Hoechst 33342 (5 ug mL^{-1}) to facilitate locating the cells in the hydrogel.

Imaging

18. Coat 24×50 mm rectangular *No. 1.5* coverglasses with 0.1 mg mL^{-1} poly-D-lysine for 10 min at r.t. and allow to dry.

19. Remove excess water carefully using laboratory wipes, and place the expanded samples on the coverglasses.

20. Set up microscope and perform imaging. In general, find the region of interest using low-magnification objectives with long working distance, and then switch to high-magnification objectives.

Note: For ExM procedure, other details could be found in the online methods and previous ExM references. For click-labeling procedure, this protocol is also applicable for other metabolically labeled biomolecules in cultured cells by changing to corresponding reagents. A few notes of click labeling are listed as follows.

1. In some cases, click labeling with alk-biotin/dye might cause higher background staining than azide-biotin/dye, enough washing with suitable surfactant (e.g., Tween 20) could solve this problem.

2. To increase the labeling efficiency of nucleic acids (e.g., metabolically labeled with EdU/EU), consider using Cu/THPTA instead of Cu/BTTAA, and try to scale up the concentration of each components.

Troubleshooting

Time Taken

The click-labeling and ExM steps could be finished within two days.