Note: This protocol has not been peer-reviewed.

Iterative expansion microscopy

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Abstract

Recently we developed iterative expansion microscopy (iExM), in which biological specimens are iteratively expanded by repeatedly embedding them in swellable hydrogels and swelling the resultant tissue-hydrogel composites. Two rounds of ~4.5x expansion result in ~4.5 x 4.5 ~20x physical magnification, enabling ~25 nm resolution imaging on conventional diffraction limited microscopes. In this protocol, we describe detailed experimental procedures starting from cultured cells or intact tissues, proceeding with immunostaining of target proteins, followed by synthesis of swellable hydrogels bearing a chemically cleavable crosslinker throughout the specimens, swelling of the gel, synthesis of a second swellable hydrogel in the space opened up by the first swelling step, swelling of the second gel, and signal amplification. This protocol has successfully been applied to cultured cells and intact tissues, enabling high precision nanoscopy on scalable, conventional diffraction-limited optics.

Introduction

We earlier showed that it is possible to physically magnify preserved biological specimens by embedding them in a densely crosslinked polyelectrolyte gel, anchoring key labels or biomolecules to the gel, mechanically homogenizing the specimen, and then swelling the gel-specimen composite by ~4.5x in linear dimension, a process we call expansion microscopy (ExM)[1–3]. The net impact is that on a conventional diffraction limited microscope with ~300 nm resolution, you can now achieve an effective resolution of ~300 / 4.5 ~ 60-70 nm. We recently developed iterative expansion microscopy (iExM), in which a sample is expanded, then a second swellable polymer mesh is formed in the space newly opened up by the first expansion, and finally the sample is expanded again (accepted, *Nature Methods*). iExM expands biological specimens ~4.5 × 4.5 or ~20x in linear dimension, and enables ~25 nm resolution imaging of cells and tissues on conventional microscopes. We have used iExM to visualize synaptic proteins, as well as the detailed architecture of dendritic spines, in mouse brain circuitry. The protocol below describes how to perform iExM. We highly recommend

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successful implementation of conventional ExM[1] before beginning work on iExM, since iExM shares many of the same steps, but applied in an iterative fashion.

Subject terms

Imaging, Neuroscience

Keywords

Microscopy, Super-resolution, Immunohistochemistry, Brain, Antibodies, Nanoscopy, STORM, PALM, STED, Expansion microscopy

Reagents

1. List of chemicals: sodium acrylate (here abbreviated AA, Sigma, 408220), acrylamide (AAm, Sigma, A9099), N,N′-methylenebisacrylamide (BIS, Sigma, M7279), ammonium persulfate (APS, Sigma, A3678), N,N,N′,N′-Tetramethylethylenediamine (TEMED, Sigma, T7024), 4-Hydroxy-TEMPO (H-tempo, Sigma, 176141), N,N'-(1,2-Dihydroxyethylene)bisacrylamide (DHEBA, Tokyo Chemical Industry, D2864), paraformaldehyde (Electron Microscopy Sciences, 15710), glutaraldehyde (Electron Microscopy Sciences 16020), Triton X-100 (Sigma, X100), glycine (Sigma, 50046), phosphate buffered saline 10x (PBS 10x, Life Technologies, 70011-044), dextran sulfate 50% (Millipore, S4030), saline-sodium citrate 20x (SSC 20x, Life Technologies, 15557), yeast tRNA (Roche, 10109495001), normal donkey serum (NDS, Jackson ImmunoResearch, 017-000-001), proteinase K (New England Biolabs, P8107S), ethylenediaminetetraacetic acid (EDTA, Sigma, EDS), guanidine HCl (Sigma, G3272), Tris-HCl, 1M pH 8.0 (Life Technologies, AM9855)
2. Blocking buffer: 1x PBS, 5% NDS, 0.1% Triton X-100
3. Hybridization buffer: 2x SSC, 10% dextran sulfate, 1 mg/mL yeast tRNA, 5% NDS, 0.1% Triton X-100
4. DNA hybridization buffer: 4x SSC, 20% formamide
5. Digestion buffer: 50 mM Tris pH 8.0, 1mM EDTA, 0.5% Triton X-100, 0.8 M guanidine HCl, 8 units/mL Proteinase K (1:100 dilution)
6. Pre-gel incubation solution: 8.625% AA, 2.5% AAm, 0.2% DHEBA, 1.865M sodium chloride, 1x PBS
7. 1st gel solution: 8.625% AA, 2.5% AAm, 0.2% DHEBA, 0.2% APS, 0.2% TEMED, 1.865M sodium chloride, 1x PBS, 0.01% H-tempo

8. Re-embedding solution: 10% AAm, 0.2% DHEBA, 0.05% APS, 0.05% TEMED
9. 2nd gel solution: 8.625% AA, 2.5% AAm, 0.15% BIS, 0.05% APS, 0.05% TEMED, 2M sodium chloride, 1x PBS
10. DNA & LNA:
    A. A1' 5'amine, AA CCG AAT ACA AAG CAT CAA CG with 5'amine
    B. A1 5'acrydite 3'alexa488, CG TTG ATG CTT TGT ATT CGG T with 5'acrydite 3'alexa488
    C. A1' 5'acrydite 3'atto565, CCG AAT ACA AAG CAT CAA CG with 5'acrydite 3'atto565
    D. A2' 4LNA-A1' 5'acrydite, GG TGA CAG GCA TCT CAA TCT ATT ACA AAG CAT CAA CGA TTA CAA AGC ATC AAC GAT TAC AAA GCA TCA ACG ATT ACA AAG CAT CAA CG with 5'acrydite
    E. LNA-A1 3'atto565, CGTTGATGCTTTGTA with 3'atto565 (underlined letters: LNA)

Procedure

1. Sample preparation

A. Cultured cells

i. Cells can be cultured as desired. In this protocol, we use Nunc Lab-Tek II chambered coverglasses (ThermoFisher, 155409).

ii. Wash cultured cells in 1x PBS three times at room temperature, briefly each time, before fixation.

iii. Fix cells with room temperature 4% formaldehyde in 1x PBS for 10 minutes.

iv. Wash cells with room temperature 100 mM glycine in 1x PBS three times, for 5 minutes each time.

B. Mouse tissue slices

i. Anesthetize mice using isoflurane in oxygen (or other animal care committee approved anesthetic) and perfuse with room temperature 1x PBS until the blood runs clear, then 30 mL room temperature fixative solution (4% paraformaldehyde in 1x PBS). Other fixation protocols may suffice as well (e.g., cold 4% paraformaldehyde in 1x PBS, perfused for 30 minutes, may work).

ii. Harvest organs of interest, then store them in the same fixative at 4°C for 24 hours.

iii. Slice organs on a vibratome (Leica VT1000s) to a thickness of 100 μm or 150

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μm, in cold 100 mM glycine in 1x PBS, then store them in 100 mM glycine in 1x PBS at 4°C until ready for staining.

2. Staining

A. Cultured cells

i. Incubate cells in blocking buffer for 10 minutes at room temperature.

ii. Incubate cells with primary antibodies in blocking buffer for one hour at room temperature and wash in 1x PBS three times for 5 minute durations each, all at room temperature.

iii. Incubate cells with DNA (e.g., A1' 5'amine)-conjugated secondary antibodies in hybridization buffer at a concentration of 10 μg/mL for one hour with gentle shaking at room temperature, then wash in 1x PBS at room temperature three times for 5 minute durations each (see http://expansionmicroscopy.org/ to find a step-by-step instruction of how to do DNA-antibody conjugation, as published in ref. [1]).

iv. Incubate cells with DNAs with 5'acrydite modification (e.g., A1 5'acrydite 3'alexa488) in hybridization buffer at a concentration of 0.5 ng/μL for one hour at room temperature with gentle shaking, then wash three times, for 5 minute durations each, in 1x PBS at room temperature.

B. Tissue slices

i. Incubate tissue slices in blocking buffer at room temperature for two hours with gentle shaking.

ii. Incubate tissue slices with primary antibodies in blocking buffer for 2-3 days at 4°C with gentle shaking and then wash in blocking buffer at room temperature with gentle shaking four times, for 30 minutes each time.

iii. Incubate tissue slices with DNA (e.g., A1' 5'amine)-conjugated secondary antibodies in hybridization buffer overnight at room temperature with gentle shaking and then wash in blocking buffer four times, at room temperature with gentle shaking, for 30 minutes each time.

iv. Incubate tissue slices with DNAs with 5' acrydite modification (e.g., A1 5'acrydite 3'alexa488) at a concentration of 1 ng/μL overnight at room temperature with gentle shaking and then wash in blocking buffer four times, at room temperature with gentle shaking, for 30 minutes each time.

3. Gelation

A. 1st gel synthesis of cultured cells

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i. Incubate cells in pre-gel incubation solution at 4°C overnight.

ii. Incubate cells in 1st gel solution at 4°C for 30 minutes.

iii. Replace the solution with a freshly prepared 1st gel solution and incubate at 4°C for 30 minutes, then incubate another three hours at 37°C.

iv. Add digestion buffer to culture wells, and then take gels out from the wells using a disposable spatula and incubate gels in digestion buffer overnight at room temperature with gentle shaking.

v. Incubate gels in DI water at room temperature with gentle shaking three times for 2 hours, 2 hours, and then overnight, respectively.

B. 1st gel synthesis of tissue slices

i. Incubate tissue slices in pre-gel incubation solution at 4°C overnight.

ii. Incubate tissue slices in fresh 1st gel solution, at 4°C for 30 minutes, twice.

iii. Place tissue slices, with accompanying 1st gel solution, between two pieces of #1 coverglass separated by another #1 coverglass (Fig. 1a) and then incubate at 37°C for 3 hours.

iv. Incubate gels in digestion buffer overnight at room temperature with gentle shaking.

v. Incubate gels in DI water at room temperature with gentle shaking three times, for 2 hours, 2 hours, and then overnight, respectively.

C. Re-embedding of expanded gels (both cultured cells and tissue slices)

i. Incubate gels in fresh re-embedding solution at room temperature for 30 minutes twice with gentle shaking.

ii. Place gels between two pieces of #1 coverglass (Fig. 1b), then place in a nitrogen-filled chamber, and incubate at 37°C for 1.5 hours.

iii. Remove from the nitrogen-filled chamber, and incubate gels in DNA hybridization buffer twice, at room temperature with gentle shaking, thirty minutes each time.

iv. Incubate gels with complementary DNA (A1' 5'acrydite 3'atto565) (or linker DNA A2' 4LNA-A1' 5'acrydite if signal amplification is desired) in DNA hybridization buffer at a concentration of 0.5 ng/μL (or 2 ng/μL for linker DNA) at room temperature overnight with gentle shaking and then wash in DNA hybridization buffer three times, at room temperature with gentle shaking, for 2 hours, 2 hours, and overnight respectively.

D. 2nd gel synthesis (both cultured cells and tissue slices)

i. Incubate gels in fresh 2nd gel solution at room temperature for 30 minutes twice

with gentle shaking.

ii. Place gels between two pieces of #1 coverglass (Fig. 1b) and then place in a nitrogen-filled chamber, and incubate at 37°C for 1.5 hours.

iii. Remove from the nitrogen-filled chamber, and incubate gels in 0.2M sodium hydroxide, at room temperature with gentle shaking, for 1 hour.

iv. For gels that will not undergo signal amplification, incubate gels in DI water three times, at room temperature with gentle shaking, for 2 hours, 2 hours, and overnight, respectively.

v. For gels that will undergo signal amplification, incubate gels in DNA hybridization buffer twice, for thirty minutes each time, at room temperature with gentle shaking, and then incubate gels with LNA (LNA-A1 3'atto565) in DNA hybridization buffer at a concentration of 0.5 ng/μL at room temperature with gentle shaking for overnight, then wash in DNA hybridization buffer at room temperature with gentle shaking three times, for 2 hours, 2 hours, and overnight, respectively. Incubate gels in DI water at room temperature with gentle shaking, three times, for 2 hours, 2 hours, and overnight, respectively.

Figure

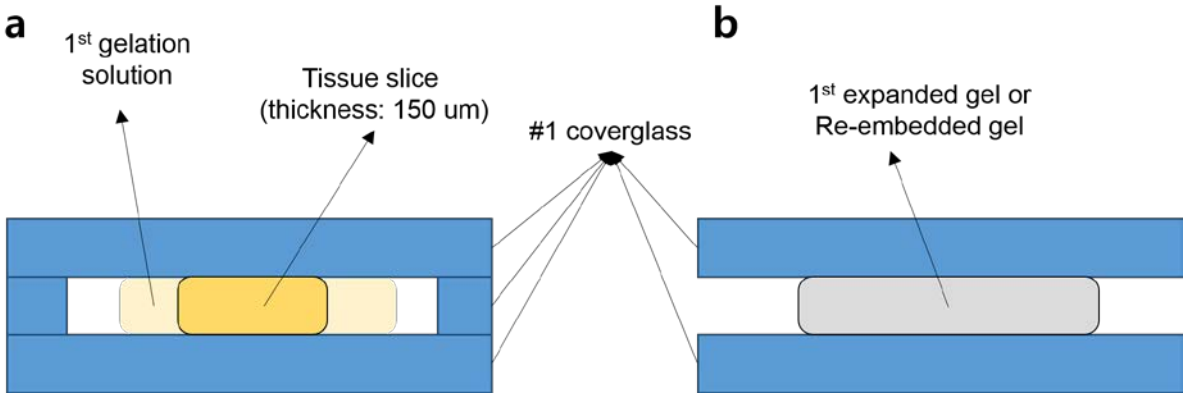

Figure 1. Assembly of gelation apparatus. (a) Gelation apparatus for 1st swellable gel synthesis, for tissue slices. (b) Gelation apparatus for re-embedding and 2nd swellable gel synthesis, for tissue slices and cultured cells.

Troubleshooting

If gels do not form, check the color of the sodium acrylate-containing solution. We make a 33% (w/w) sodium acrylate stock solution and use it to make the final gel solutions. The sodium acrylate stock solution should be colorless (or slightly yellow), but not very yellow – if very yellow, that means the sodium acrylate has gone bad. We recommend storing sodium acrylate

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powder at -20^{o}C.

References

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2. Tillberg, P. W. *et al.* Protein-retention expansion microscopy of cells and tissues labeled using standard fluorescent proteins and antibodies. *Nat. Biotechnol.* **34,** 987–992 (2016).
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Associated publications

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Competing financial interests

E.S.B., J.-B.C., F.C., and P.W.T. have applied for a patent on iExM. E.S.B. is co-founder of a company, Expansion Technologies, that aims to provide expansion microscopy kits and services to the community.

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