Expansion microscopy of cellular and bacterial membranes by functionalized ceramides

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Abstract

Due to the lack of primary amino groups, lipids cannot be fixed by formaldehyde, glutaraldehyde and other chemical fixatives nor expanded using expansion microscopy (ExM). We present a protocol that uses unnatural short-chain azide- and amino-functionalized ceramides, which upon incorporation into cellular and bacterial membranes can be labeled by click chemistry and linked into hydrogels, followed by 4x to 10x expansion.

Introduction

Expansion microscopy (ExM) enables super-resolution imaging on standard fluorescence microscopes. By linking a protein of interest into a dense, cross-linked network of a swellable polyelectrolyte hydrogel, biological specimens can be physically expanded allowing ~70 nm lateral resolution by confocal laser scanning microscopy. Since its introduction by Boyden and co-workers in 2015¹, expansion microscopy (ExM) has shown impressive results including the magnified visualization of pre- or post-expansion labeled proteins and RNAs with fluorescent proteins, antibodies, and oligonucleotides, respectively, in cells, tissues, and human clinical specimen². In order to be usable for ExM, the molecule of interest has to exhibit amino groups that can react with glutaraldehyde (GA)³, MA-NHS³, AcX⁴, or Label-X⁵ and be linked into the polyelectrolyte hydrogel. The plasma membrane of cells is mainly composed of glycerophospholipids, sphingolipids, and cholesterol. Due to the lack of primary amino groups, these lipids neither can be fixed by formaldehyde, glutaraldehyde and other chemical fixatives nor expanded using available ExM protocols. We used an unnatural short-chain sphingolipid functionalized with an azide and primary amino group to enable fluorescence labeling and chemical fixation as well as linking of the lipid into a swellable hydrogel. Our results demonstrate that the designed unnatural bifunctional sphingolipid is efficiently incorporated into membranes of cells and accumulates in bacterial membranes, which allowed us to investigate the distribution of lipids and interactions with proteins in cellular and bacterial membranes with high spatial resolution.

Reagents

- α -NH₂- ω -N₃-C₆-ceramide (homemade)
- 1x PBS (Thermo Fisher, 14190169)
- 4% Paraformaldehyde (PFA, Morphisto, 10303.01000)
- Glutaraldehyde 25% (GA, Sigma Aldrich, G5882)
- Triton X-100 (Thermo Fisher, 28314)
- Hanks' Balanced Salt solution (HBSS, Merck, H6648)

- Click-IT Alexa Fluor® 488 DIBO alkyne dye (Jena Bioscience, CLK-1278-1)
- Fetal bovine serum (FBS, Sigma-Aldrich, F7524)
- Ammonium persulfate (APS, Sigma, A3678)
- Tetramethylethylenediamine (TEMED, Sigma, T7024)
- Protease K (Thermo Fisher, AM2548)
- Lysozyme (BioChemika, 62970)
- Poly-D-Lysin (PDL, Gibco, A3890401)
- Sodium acrylate (Sigma, 408220)
- 2.5 % Acrylamide (Sigma, A9926)
- 0.15 % N,N'-Methylenbisacrylamide (Sigma, A9926)
- 2 M NaCl (Sigma, S5886)
- DMAA (Sigma, 274135)
- KPS (Sigma, 379824)
- EDTA (Sigma, ED2P)
- Guanidine HCl (Sigma, 50933)

Reagent setup

For preparation of 4x monomer solution mix:

- 8.625 % Sodium acrylate (Sigma, 408220)
- 2.5 % acrylamide (Sigma, A9926)
- 0.15 % N, N'-Methylenbisacrylamide (Sigma, A9926)
- 2 M NaCl (Sigma, S5886)

dissolved in 1x PBS.

For preparation of 10x monomer solution mix:

- 1 ml of 10x Monomer solution contains
 - 0.267 g DMAA (Sigma, 274135)
 - 0.064 g Sodium acrylate (Sigma, 408220)
- dissolved in 0.57 g ddH₂O

The digestion buffer consists of

- 50 mM Tris pH 8.0
- 1 mM EDTA (Sigma, ED2P)
- 0.5 % Triton X-100 (Thermo Fisher, 28314)
- 0.8 M Guanidine HCl (Sigma, 50933)

Equipment

- 15 mm glass cover slide (VWR, ECN 631-1579)
- Costar 12 Well Cell Culture plate (Corning, 3513)
- Parafilm (Hartenstein, PF10)
- TC dish 150 (Sarstedt, 83.3903)
- Glass chambers (Merck, 734-2055)

Procedure

Sample preparation

Day 1:

1. Grow cells overnight on a 15mm glass cover slide in a 12-well plate in a humidified atmosphere containing 5% (v/v) CO₂ at 37°C.

Day 2:

2. *Optional: For sphingolipid ExM of bacteria infect the cells according to standard protocols and follow the protocol after bacterial propagation.*

Note: Ceramides may exhibit toxic effects on some bacterial species.

3. Exchange the media of the cells for fresh media prewarmed to 37°C
4. Treat the cells with 10 µM α -NH₂- ω -N₃-C₆-ceramide prewarmed to 37°C for 1 hour at 37°C in the exchanged medium
5. Wash the sample with 1x PBS
6. Fix the cells using 4% PFA and 0.1% GA for 10 minutes
7. Wash the sample 3 times with 1x PBS

Click-reaction

8. Permeabilize the sample for 15 minutes in 0.2% Triton X-100 in 1x PBS
9. Wash the sample 3 times with 1xPBS
10. Exchange the PBS for 1x HBSS prewarmed to 37°C
11. Incubate the sample in 5 µM Click-IT Alexa Fluor® 488 DIBO alkyne dye at 37°C for 30 minutes. *Note: Other functionalized dyes can be used according to preferences.*
12. Wash the sample 3 times with 1x PBS

Optional: Immunostaining

For samples that need additional immunolabelling, e.g. for colocalization of cellular or bacterial proteins, follow the steps a-e before proceeding with step 13.

- a. Block the sample with 2% FBS in 1x PBS for 1 hour.*
- b. Incubate the sample in the primary antibody diluted in blocking buffer for 1 hour in a humid chamber*
- c. Wash the sample 3 times with 1x PBS*
- d. Incubate the sample in the corresponding secondary antibody diluted in blocking buffer for 1 hour in a humid chamber*

Note: Not all secondary antibodies are suitable for expansion microscopy. Our preferred secondary antibodies are Alexa Fluor 488, CF568, Atto643 and Atto647N.

- e. Wash the sample 3 times with 1x PBS*

Expansion Microscopy

13. Incubate the labelled sample with 0.25% GA at RT for 10 minutes.

14. Wash the sample 3 times with 1x PBS

Note: The monomer solutions for 4x and 10x Expansion are different.

15. **For 4x Expansion**, add 0.2% ammonium persulfate (APS) and 0.2% tetramethylethylenediamine (TEMED) to the 4x monomer solution

16. Immediately pipet 80 µl of the mixture on parafilm and turn the labeled 15 mm glass slide with the cells facing the bottom onto the drop

17. Incubate for 1 hour at RT for gelation

18. **For 10x Expansion**, degas 1 ml of the 10x monomer solution on ice for 45 minutes with nitrogen.

19. Add 100 µl KPS (0.036 g/ml) to the monomer solution

20. Degas on ice for another 15 minutes with nitrogen

21. Add 4 µl of TEMED

22. Immediately pipet 80 µl of the mixture on parafilm and turn the labeled 15 mm glass slide with the cells facing the bottom onto the drop

23. Incubate for 30 minutes at RT for gelation, followed by 1.5 hours at 37°C

24. After gelation, remove the gel from the glass cover slide and digest the samples between 3 hours and overnight with 8U/ml protease K in digestion buffer

*Note: The cell wall digestion of some bacteria requires additional treatment, e.g. 1mg/ml lysozyme in the case of *Neisseria gonorrhoeae*.*

25. Coat glass chambers with PDL overnight at RT.

Day 3:

26. Incubate digested gels in hourly changed ddH₂O for expansion until expansion is saturated

Note: Due to the swelling of the gel, make sure that the fully expanded gel has enough space in the dish used, e.g. TC dish 150.

27. Chop expanded gels in pieces fitting the PDL-coated glass chambers for imaging

Troubleshooting

Time Taken

Anticipated Results

The described protocol enables nanoscale imaging of cellular and bacterial membranes and differentiation of both layers within double membranes.

References

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Figures

Figure 1

Sphingolipid ExM enables super-resolution imaging of cellular membranes. Confocal fluorescence image of 4x expanded HeLa229 cells, infected with *C. trachomatis*, fed with α -NH₂- ω -N₃-C₆-ceramide, clicked with DBCO-Alexa Fluor 488 (green) and immunolabeled for chlamydial HSP60 (magenta). Scale bar, 20 μ m.

Figure 1

Sphingolipid ExM enables super-resolution imaging of cellular membranes. Confocal fluorescence image of 4x expanded HeLa229 cells, infected with *C. trachomatis*, fed with α -NH₂- ω -N3-C6-ceramide, clicked with DBCO-Alexa Fluor 488 (green) and immunolabeled for chlamydial HSP60 (magenta). Scale bar, 20 μ m.