**Expansion microscopy**

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**ABSTRACT**

Expansion microscopy is a technique to visualize biological structures with higher spatial resolution than traditional microscopy methods.

**Protocol**

Expansion microscopy (ExM) was performed as described 60 with some modifications. Cells were blocked with 10% (v/v) normal goat serum (NGS) in 0.1% (v/v) Triton X-100 in PBS and incubated with primary antibodies in blocking solution overnight. After a 3-h incubation with the corresponding secondary antibody (Alexa Fluor, Invitrogen), the samples were washed and treated with 0.1 mg/ml Acryloyl-X SE solution (Thermo Scientific) in PBS for 3 h at room temperature. The freshly prepared gelling solution consisted of Stock X solution (8.6% (w/v) sodium acrylate 33% (w/v), 2.5% (w/v) acrylamide 50% (w/v), 0.15% (w/v) N,N´-methylenebisacrylamide 2% (w/v), 11.7% (w/v) NaCl 5 M, and PBS 1X), water, 10% (w/v) TEMED and 10% (w/v) APS stock solution in a 47:1:1:1 ratio. Gel digestion was performed overnight in digestion buffer (0.5% (w/v) Triton X-100, 0.2% (v/v) EDTA 0.5 M, pH 8, 5% (v/v) Tris-Cl 1 M, pH 8, 4.67% (w/v) NaCl and 8 U/ml proteinase K). The gelling solution was added to each well and covered by a 15-mm coverslip to ensure the formation of a smooth, flat and thin gel. Coverslips were then incubated for 1 h at 37°C for complete polymerization. The gel was expanded in water for 1 h and mounted in 10 µg/mL poly-L-ornithine-coated coverslips to immobilize the gel for picture acquisition. Midbrain organoids were fixed, and immunofluorescence staining was performed as described above. Sections were treated with 0.1 mg/ml acryloyl-X SE solution in PBS at room temperature overnight. Gelation was performed in a 47:1:1:1 ratio of Stock X, 10% (w/v) TEMED, 10% (w/v) APS, and 0.5% (w/v) 4-hydroxy-TEMPO stock solutions. Gel digestion and expansion were performed as described above. Images were acquired using a Leica TCS SP8 confocal microscope (Leica, Germany) equipped with a 100× /1.4 numerical aperture oil-immersion objective. For each condition, 5 images were acquired from at least three independent experiments. Images were analyzed using Diffraction PSF 3D, DeconvolutionLab2, and EzColocalization plugins in Fiji-ImageJ. GraphPad Prism version 9.0.0 (RRID:SCR\_002798) was used to calculate Spearman’s rank correlation value (ρ) to identify colocalization of fluorescence signals.