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mFISH3D-2.0 V.1

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We use this protocol and it's working

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Abstract

The protocol is for the multiplexed in situ hybridization in 3D (mFISH3D-2.0) in an adult mouse brain / a block of a human brain. A comprehensive upgrade of old mFISH3D (https://www.protocols.io/view/mfish3d-kqdg3pjxql25/v1), representing a new generation rather than a simple extension.

Guidelines

See safety data sheets for proper chemical handling, precautionary measures, and waste disposal.
Obey all local regulations/guidelines for handling and disposal of used reagents and solutions containing reagents mixed in.

Materials

To prepare a lithium maleate buffer, add lithium hydroxide to a maleic acid solution until the pH reaches 7.0. It is recommended to prepare a 1 M lithium maleate buffer stock solution.

Material table

	A	B	C
	16% PFA	Electron Microscopy Sciences	Cat. # 15710
	10X PBS	Invitrogen	Cat. # AM9625
	Proteinase K	Ambion	Cat. # AM2544
	Methanol	Fisher Scientific	Cat. # A412SK
	Formamide	Ambion	Cat. # AM9342
	Poly(ethylene Glycol) 20,000	Sigma	Cat. # 95172
	Maleic acid	Sigma	Cat. # M0375
	Lithium hydroxide	Sigma	Cat. # 442410
	Sodium chloride	Millipore	Cat. # 567440
	Lithium chloride	Sigma	Cat. # 213233
	Hydrogen peroxide solution	Sigma	Cat. # 516813

	A	B	C
	Benzyl alcohol	Sigma	Cat. # 24122-1L
	Benzyl benzoate	Sigma	Cat. # W213802-5KG-K
	Benzyl acetate	Sigma	Cat. # B15805

Troubleshooting

Safety warnings

Formamide:
Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution and dispose of waste appropriately.
Suspected of causing cancer.
May damage fertility or the unborn child.
May cause damage to organs (Blood) through prolonged or repeated exposure if swallowed.

Formaldehyde/paraformaldehyde/formalin solution (PFA):
Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution and dispose of waste appropriately.
May cause cancer.
Toxic if swallowed, in contact with skin or if inhaled.
Causes severe skin burns and eye damage.
May cause an allergic skin reaction.
May cause respiratory irritation.
Suspected of causing genetic defects.
Causes damage to organs (Eyes).

Methanol:
Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution and dispose of waste appropriately.
May be fatal or cause blindness if swallowed. Vapor harmful. Flammable liquid and vapor. Harmful if swallowed, inhaled, or absorbed through the skin. Causes eye, skin, and respiratory tract irritation. May cause central nervous system depression. Cannot be made non-poisonous.

Tissue sample preparation: mouse brain, block of a fresh frozen human brain

6h

1 If the sample is a fresh frozen sample and requires sub-dissection, bring the tissue at -40 °C for more than 03:00:00 . This enables the sub-dissection of the sample using a regular disposable blade on dry ice.

2 If the sample is frozen, bring the tissue at -20 °C for more than 03:00:00 .
This reduces the risk of the formation of ice crystals when moved to a fixation solution.

3 Gently shake tissue in 40 mL 4% paraformaldehyde in PBS Overnight at 4 °C . The amount of PFA should be adjusted based on the size of the tissue. It is usually advised to use at least 10 times the volume of the tissue sample.

4 (Optional) Subdissect the tissue. The dissected volume will be the final volume.

5 Initiate dehydration by immersing the tissue in a gradient concentration of methanol (MeOH). 6h
The volume of the solution is the same as the volume used for PFA fixation.
Replace the solution with 80% MeOH. Gently shake the sample at 4 °C for 02:00:00 .

Refresh 80% MeOH. Gently shake the sample at 4 °C for 02:00:00 .

Replace the solution with 100% MeOH. Gently shake the sample at 4 °C for 02:00:00 .

Refresh 100% MeOH. Gently shake the sample at Room temperature Overnight .

6 Refresh 100% MeOH. Gently shake the sample at 50 °C for 120:00:00 (5 days).
Refresh MeOH every day but can be skipped during a weekend or national holidays. If necessary, the delipidated tissues can be stored at -20°C until use.

(Optional) Photobleaching of autofluorescence

10h

7 4h

Though optional in rodent tissue, photobleaching will significantly improve the signal-to-noise ratio. This step is a must for human tissue.

Depending on the level of autofluorescence, prepare
#1, BABB, or
#2, [M] 0.2 % volume H_2O_2 in BAcBB.
BABB can be prepared by mixing benzyl alcohol and benzyl benzoate in a 1:2 ratio.
BAcBB can be prepared by mixing benzyl acetate and benzyl benzoate in a 1:1 ratio.
To mix H_2O_2 in BAcBB, add the required amount of H_2O_2 to Benzyl acetate, and very gently shake the solvent until fully mixed (Do NOT vortex!). Then add benzyl benzoate and mix.
#2 is preferred for tissue with strong autofluorescence, such as human tissue.
Otherwise, use #1.

Use 3 mL for a whole mouse brain or 2 mL if the tissue is smaller than that.
After 01:00:00 of shaking in the cleaning solution, refresh the solution and keep the tissue shaken until fully cleared. This usually takes 03:00:00 for a whole brain.

Once the tissue gets cleared, start photobleaching under strong LED illumination.
A large photobleaching device can be fabricated by referring https://github.com/NBelenko/Photobleacher.
If not, use an LED from Thorlabs (MWWHLP1) with an adjustable collimation adapter (SM1U25-A) and give max-power illumination from a 2-cm distance.

Illuminate the tissue Overnight.

8 6h

Wash out BABB by immersing the tissue in 100% MeOH.
Gently shake the tissue at Room temperature 02:00:00.
Refresh 100% MeOH. Gently shake the tissue at Room temperature 02:00:00.
Refresh 100% MeOH. Gently shake the tissue at Room temperature 02:00:00.

If necessary, the delipidated tissues can be stored at -20°C until use.

Pre-processing

4h 30m

9 1h

We use a 5 mL tube with a screw cap for a whole-mouse brain or a 2 mL Eppentube for smaller tissue.

1.5 mL of solution is used for a whole-mouse brain or 1 mL of solution for smaller tissue.

10 **(Optional)** 3h 30m

We found that this protease step introduces significant variability depending on the size of tissue, the manufacturer, or batch of proteinase K. Therefore, we no longer recommend performing this proteinase K step, and it is entirely optional. You may proceed directly to the primary hybridization step following the MeOH wash described above.

Transfer tissue in MeOH to MeOH with 2.5 M LiCl.
Gently shake the tissue for 01:00:00 at Room temperature .

Mix 10 ug/mL of proteinase K in primary hybridization buffer (1 M NaCl, 40% formamide in 100 mM lithium maleate buffer (pH 7.0)).

Place the tissue in the proteinase K solution, and gently shake the tissue for 03:00:00 at Room temperature .

Wash tissue with primary hybridization buffer at Room temperature . Wash for 00:30:00 and repeat the process in total twice.

Primary probe hybridization 2d 2h

11 1.5 mL of solution is used for a whole-mouse brain or 1 mL of solution for smaller tissue. 2d
Replace the solution with hybridization buffer with primary probes.
Shake the sample gently at 37 °C for 48:00:00 or 2 nights. This can be 3 nights, depending on the weekend schedule.

For the concentration of the primary probe, I advise beginning with [M] 4.0 nanomolar (nM) per each oligonucleotide.
If you see the limited penetration of the HCR probes, you should reduce the concentration of the primary probe. The primary probe penetrates evenly regardless of

the concentration. Too high concentration of primary probes limits the penetration of HCR probes by trapping HCR probes at the rim.
Check out my GitHub repository (https://github.com/tatz-murakami/split-oligo-designer) for the design of the primary probes.

12 Wash the tissue with pre-HCR washing buffer (1 M NaCl and 20% formamide in 100 mM lithium maleate buffer (pH 7.0)) by gently shaking for 02:00:00 three times before the HCR amplification. 2h

HCR probe hybridization 2d 2h

13 For HCR amplification, use 900 µL for a whole-mouse brain, or 600 µL for smaller tissue. 2d
For washing, use 1.5 mL or 1.0 mL .

Place the tissue in HCR buffer (1 M NaCl, 20% formamide, and 10% PEG(20000) in 100 mM lithium maleate buffer (pH ~7.0)) with 30 nM of HCR probes.
The HCR probes are denatured prior to the reaction by heating the probes to 95°C and leaving them at RT for at least 30 minutes.

Perform HCR reaction at 37 °C for 48:00:00 or 2 nights. This can be 3 nights, depending on the weekend schedule.
If you see the dim signals, extend the duration for 4 days with 60 nM of HCR probes.

14 Wash the sample with post-HCR washing buffer (0.5 M LiCl, 40% formamide in 100 mM lithium maleate buffer (pH 7.0)) at Room temperature with a gentle shake for 02:00:00 three times. 2h

In our original manuscript, we used 1M LiCl at 37 °C, but we found that 0.5 M LiCl at room temperature better preserves the signal without compromising the RNA quality. Please use the washing buffer described above.

Tissue clearing 1h

15 1.5 mL of solution is used for a whole-mouse brain or 1 mL of solution for smaller tissue. 1h

Place the tissue in pre-clearing washing buffer (2.5 M LiCl in 100 mM lithium maleate buffer (pH 7.0)).
The tissue is gently shaken at Room temperature Overnight .

Replace the solution with 2.5 M LiCl MeOH solution.
Gently shake the sample for 00:30:00 at Room temperature .
Refresh 100% MeOH.
Gently shake the sample for 00:30:00 at Room temperature .
Refresh 100% MeOH.
Gently shake the sample for 00:30:00 at Room temperature .

Bring the tissue to BABB.
Gently shake the sample for 01:00:00 at Room temperature .
Repeat the process until the tissue becomes transparent. Usually, a whole brain can be cleared in three hours.

Imaging

16 Image the sample as you like! I highly recommend the use of light-sheet microscopy, which is designed for cleared tissue. I suggest the use of BABB as an immersion medium, but it is also possible to perform imaging using an immersion oil mixture, a 3:1 mixture of HIVAC-F4 (Shin-Etsu, refractive index = 1.555) and HIVAC-F5 (Shin-Etsu, refractive index = 1.575), if there is a need to avoid using BABB as the immersion medium.

Photobleaching (Only for multi-round staining)

17 Please go to go to step #7 . Photobleach with H_2O_2 to bleach the fluorescent dyes.