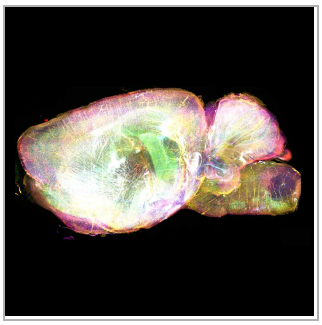

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WORKS FOR ME 1

mFISH3D

COMMENTS 0

DOI
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ABSTRACT

The protocol is for the multiplexed in situ hybridization in 3D (mFISH3D) in an adult mouse brain / a block of a human brain.

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KEYWORDS

in situ hybridization, hybridization chain reaction, tissue clearing, BABB, light-sheet microscopy

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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 TEXT

16% paraformaldehyde
UltraPure 20X SSC buffer (Invitrogen)
(optional) UltraPure Distilled Water (Invitrogen)

Citric acid
Tween-20
6 M HCl
Benzyl alcohol
Benzyl benzoate
Methanol
Proteinase K

Oligonucleotides, primary probes
Oligonucleotides, HCR probes

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.

Hydrochloric acid (HCl):
Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution and dispose of waste appropriately.
May be corrosive to metals.
Causes severe skin burns and eye damage.
May cause respiratory irritation.

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.

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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.

BEFORE STARTING

If you worry about the contamination of RNAse, use RNAse-free water to make all the reagents.

1d 22h 30m

Tissue sample preparation: a whole-mouse brain, blocks of a fresh fr

16h

1 Incubate tissue in 4% paraformaldehyde (pH 6.5~7.0) overnight at 4°C.

Overnight

Note

We avoid the pH of the PFA solution to be more than 7.5. Though it is known that higher pH can more solubilize PFA, the basic pH can degrade mRNAs. We use pre-made 16% PFA (#15710, Electron Microscopy Sciences). Dilute the 16% PFA to 4% in PBS. Add 13.3 ul of 6 M HCl to 40 ml of the solution to make pH ~6.5.

2 (optional) Subdissect the tissue. The dissected volume will be the final volume.

1h

3 For the following sample preparation steps, use 4 ml of solution per one tissue block.
I recommend using a 5 ml Eppentube or 15 ml Falcon tube.

Wash the tissue in 2xSSC, 4 mM citric acid. Gently shake at 4°C.
00:30:00
Refresh 2xSSC 4 mM citric acid solution. Gently shake at 4°C.
00:30:00

Note

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If you are going to do photobleaching, I encourage you to wash the tissue longer 01:00:00 each.
Otherwise, you will see PFA/salt precipitation after clearing.

1d 2h 30m

4 Start dehydration by immersing the tissue in gradient concentrations of methanol (MeOH).
Replace the solution with 30% MeOH. Gently shake at RT.
00:30:00

Replace the solution with 50% MeOH. Gently shake at RT.
00:30:00

Replace the solution with 80% MeOH. Gently shake at RT.
00:30:00

Replace the solution with 100% MeOH. Gently shake at RT.
00:30:00

Refresh 100% MeOH. Gently shake at RT.
00:30:00

Refresh 100% MeOH. Move the tissue to 37°C and keep gentle shakes.
Shake the tissue for 16-48 hrs depending on the tissue size.
24:00:00

5 Refresh 100% MeOH.
For long-term storage, move the tissue to -20°C. I confirmed RNA is stable for at least 6 months.

3h

6 (optional) The following few steps are optional.
This step is for photobleaching to quench autofluorescence.
The photobleacihng is critical for human brain imaging while you can skip this step for rodent brains.

Replace the solution with BABB. Gently shake at RT until the tissue gets cleared.
03:00:00
If the transparency is not high, refresh BABB and gently shake the tissue for additional hours.
Move the tissue to a glass cuvette or polypropylene plastic container.

Note

Beware BABB is not compatible with polystyrene plastic, a common plastic for cell culture dishes.

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2d

7 Photobleach the tissue with a bright LED.
I use the LED from Thorlabs (MWWHLP1) with an adjustable collimation adapter (SM1U25-A) and give max-power illumination from a 2-cm distance.
Illuminate the tissue in BABB for one or two nights until you do not see autofluorescence.
48:00:00

Note

If you choose to build a custom-made photobleacher, please be aware of the following factors.
1, wavelength spectrum (usually white LED lacks blue color illumination)
2, power and flux
3, cooling

1h 30m

8 Wash out BABB by immersing the tissue in 100% MeOH.

Gently shake the tissue at RT.
00:30:00

Refresh 100% MeOH. Gently shake at RT.
00:30:00

Refresh 100% MeOH. Gently shake at RT.
00:30:00

Refresh 100% MeOH.
For long-term storage, move the tissue to -20°C. I confirmed RNA is stable for at least 6 months.

22h

Preprocessing

18h 30m

9 Unless noted, use a 5 ml Eppentube for tissue processing.

Equilibrate the brain in refreshed 100% MeOH at RT.
00:30:00
Replace the solution with 80% MeOH. Gently shake at RT.
00:30:00
Replace the solution with 50% MeOH. Gently shake at RT.
00:30:00
Replace the solution with 30% MeOH. Gently shake at RT.
00:30:00
Replace the solution with 4 ml of 10 mM HCl. Gently shake at 37°C.
00:30:00
Refresh 10 mM HCl. Gently shake at 37°C overnight.

Citation: Tatsuya Murakami mFISH3D https://dx.doi.org/10.17504/protocols.io.kqdg3pjxql25/v1

16:00:00

3h 30m

10

Rinse the sample with wash buffer 1 (5xSSC, 20 mM citric acid, 0.01% Tween-20) briefly.
Wash the tissue with wash buffer 1 at RT with a gentle shake.
00:30:00
Replace the solution with 2 ml of 10 ug/ml proteinase K in washing buffer 2 (2xSSC, 4 mM citric acid, 0.01% Tween-20).
Shake the tissue at RT for 2 - 5 hours depending on the tissue size.
02:00:00
Wash the sample with wash buffer 1 at RT with a gentle shake.
00:30:00
Refresh wash buffer 1. Gently shake at RT.
00:30:00

1d 1h 30m

Primary probe hybridization

19h 30m

11

Use a 2 ml Eppentube to save the oligo probes.
If the sample does not fit a 2ml tube, use a 5 ml tube with double the amount of the solution.

Move the tissue to 500 ul of hybridization buffer (30% formamide, 5xSSC, 9 mM citric acid, (optionally 3% PEG8000)) for pre-hybridization. Shake gently at RT.
00:30:00
Replace the solution with 500 ul of hybridization buffer with [M] 1 micromolar (µM) See the note below primary probes. Shake gently at 37°C.
I confirmed the hybridization in a half hemisphere brain can be done in 3 hrs. For a whole mouse brain, I recommend longer incubation up to 16 hrs.
03:00:00 or 16:00:00

Note

The concentration of the primary probe is a **total concentration** of oligos. If you have 50 oligonucleotides in the solution, the concentration of each oligo should be 20 nM.
It is convenient to make 1 mM of primary probes as a stock solution.
Check my GitHub repository (https://github.com/tatz-murakami/split-oligo-designer) for the design of the primary probes.

6h

12

Wash the tissue with wash buffer 1. Wash three times in total, 2 hours for each.
02:00:00

Citation: Tatsuya Murakami mFISH3D https://dx.doi.org/10.17504/protocols.io.kqdg3pjxql25/v1

02:00:00
02:00:00

Note

Occasionally, you see the non-specific signal after clearing. The non-specific signal can be suppressed by adding stringency at this washing step. If you see the non-specific signal, add 10% formamide in washing buffer 1 in this step. You do not have to add formamide in other washing steps.

1d 7h

HCR probe hybridization

30m

13 Equilibrate the tissue with 500 ul of hybridization buffer for pre-hybridization. Shake gently at RT.
00:30:00

30m

14 During the pre-hybridization, denature HCR probes as follow.
Prepare necessary amounts of HCR probes in PCR microtubes.
Heat the PCR tubes at 95°C for a minute using a thermal cycler.
Remove the tubes from the cycler, and cool down the tubes at RT for ~30 minutes in a dark space.
00:30:00

Note

I prepare 30 µM of stock solution of HCR probes in a hybridization buffer. To avoid evaporation in the PCR tube, add a hybridization buffer up to 10 µl.
You can either buy HCR probes from Molecular Instruments or synthesize HCR probes from a vendor such as Integrated DNA Technologies.
If you choose to synthesize HCR probes, choose the dual HPLC purification or HPLC+PAGE purification.

1d 6h

15 Replace the solution with 500 ul of hybridization buffer with 90 nM of HCR probes.
Shake gently at RT for more than 16 hours.
For a whole-mouse brain, incubation of more than 30 hours is recommended.
30:00:00

12h 30m

Tissue clearing

Citation: Tatsuya Murakami mFISH3D https://dx.doi.org/10.17504/protocols.io.kqdg3pjxql25/v1

16 Wash the tissue with wash buffer 1. Wash twice in total. 1 hour for each. 4h

01:00:00

01:00:00

For desalting, replace wash buffer1 with wash buffer 2. Wash twice in total. 1 hour for each.

01:00:00

01:00:00

Note

Wash buffer 2 is a low-salt-concentration buffer. If you skip this step, you get uncleared tissue after RI matching.
While wash buffer 1 can retain hybridization, wash buffer 2 can cause de-hybridization if incubated too long. Limit the duration of the incubation to 2-3 hours.

8h 30m

17 For dehydration, you can either use a 2 ml tube or a 5 ml tube.

Replace the solution with 30% MeOH. Gently shake at RT.

00:30:00

Replace the solution with 50% MeOH. Gently shake at RT.

00:30:00

Replace the solution with 80% MeOH. Gently shake at RT.

00:30:00

Replace the solution with 100% MeOH. Gently shake at RT.

00:30:00

Refresh 100% MeOH. Gently shake at RT.

00:30:00

Replace the solution with BABB. Gently shake at RT.

03:00:00

Refresh BABB and gently shake the tissue for additional hours until the tissue gets cleared.

03:00:00

Congratulations! The tissue is now ready to be imaged.
Please follow the next step if you want additional cycles.

6h

Dehybridization/Photobleaching

18 If you designed the experiment in a way that all the primary probes from different cycles do not have cross-talk, photobleaching is more recommended than dehybridization.
If not, you have to remove the primary probes from the previous cycle.

If you choose to photobleach, check step 7 and ignore the dehybridization step 21.

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go to step #7

1h 30m

19 Wash out BABB by immersing the tissue in 100% MeOH.

Gently shake the tissue at RT.
00:30:00

Refresh 100% MeOH. Gently shake at RT.
00:30:00

Refresh 100% MeOH. Gently shake at RT.
00:30:00
(Optional pausing point) You can keep washing in MeOH overnight. Store the sample at -20°C for a long term.

2h 30m

20 Replace the solution with 80% MeOH. Gently shake at RT.
00:30:00
Replace the solution with 50% MeOH. Gently shake at RT.
00:30:00
Replace the solution with 30% MeOH. Gently shake at RT.
00:30:00
Replace the solution with wash buffer 1. Gently shake at RT.
00:30:00
Move the tissue to 500 ul of hybridization buffer for pre-hybridization. Shake gently at RT.
00:30:00

2h

21 Replace the solution with 500 ul of hybridization buffer with 1 µM primary probes.
Move the tissue to 37°C. Gently shake the tissue.
01:00:00
Move the tissue to 65°C. Incubate the tissue for 1 hour.
01:00:00

22 Refresh the solution with 500 ul of hybridization buffer with 1 µM primary probes.
Move the tissue to 37°C. Gently shake the tissue for the duration shown in step 11.

Follow the left HCR and clearing steps as described above.
go to step #12

Citation: Tatsuya Murakami mFISH3D https://dx.doi.org/10.17504/protocols.io.kqdg3pjxql25/v1