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# Highly Amplified Multiplexed Fluorescence In Situ Hybridization (hamFISH)

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hamFISH

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**We use this protocol and it's working**

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## Disclaimer



## Abstract

**This is the protocol associated with *Combined transcriptomic, connectivity, and activity profiling of the medial amygdala using highly amplified multiplexed in situ hybridization (hamFISH)***
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In the main report we describe the development of hamFISH and its use in spatially profiling neurons in the medial amygdala of mice. This protocol describes how to perform hamFISH on non-fixed mouse brains, with notes of probe design, probe synthesis and sequential imaging included.

## Attachments

XLSX

**hamFISH amplifier se...**

17KB

## Materials

**MATERIALS FOR PROBE SYNTHESIS**

PCR amplification
Kapa HiFi Polymerase (Roche, KR0369)

*In vitro* transcription
HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, E2040)

Reverse transcription
Maxima H Minus Reverse transcriptase (Thermo Fisher, EP0751)

Column purification
ENZA Plasmid DNA kit columns(Omega Biotek, D6943)

**MATERIALS FOR TISSUE PREPARATION AND HYBRIDIZATION**

Coverslip and coverslip preparation
40 mm diameter #1.5 coverslips (Bioptechs, 0420-0323-2)
(3-mercaptopropyl)trimethoxysilane (Sigma, 175617)
poly-L-lysine (Sigma, P8920)

Fixation solution
4% formaldehyde,
0.4% glyoxal (Sigma, 50649)
0.1% methanol,
1xPBS

100% Ethanol
Store aliquot at -20C for at least one hour before use

2x SSC and 0.2xSSC
Diluted from 20x SSC (Severn Biotech)

4% SDS
Prepare 50 mL with 2g SDS, 5 mL 10xPBS, top up to 50 mL with H2O

Protease (Sigma, P5380)

Pre-hyb buffer
2xSSC with 22.5% formamide (Sigma, F7503)

Hyb buffer
Mix 1:1 of RCA buffer with smFISH buffer (see below)

RCA buffer
2xSSC, 10% formamide (Sigma, F7503), 1% tween-20 (VWR, 437082Q), 20mM RVC (New England Biolabs, S1402), 0.1% wt/v yeast RNA (ribonucleic acid from Torula utilis, Sigma, 83850).
RVC frozen in 50 ul aliquots at 200mM. When preparing RCA hyb buffer, thaw at 65C before adding 50 ul to 450 ul RCA hyb buffer.

smFISH buffer
2xSSC, 10% dextran sulphate (Millipore, S4031), 35% formamide

Amplification buffer
2xSSC, 10% dextran sulphate, 20% formamide

Amplifier wash buffer
2xSSC with 20% formamide

Monomer buffer
2x SSC
4% acrylamide
0.2% bis-acrylamide

Digest buffer
2xSSC, 2%SDS, 0.5% tritonX (Sigma §X100), 1:20 proteinaseK (20mg/ml, (Ambion, AM2548)).

DAPI solution
500ng/mL DAPI in 2xSSC

Fluidics hyb buffer
2xSSC, 10% dextran sulphate, 40% formamide

**SEQUENCES FOR AMPLIFICATION AND SIGNAL DEVELOPMENT**

All sequences can be found in the attached table.

## Summary

1

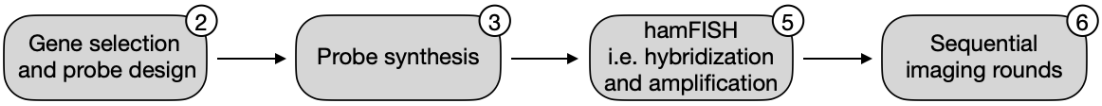


Figure 1: hamFISH workflow. Numbers refer to sections below.

## Gene selection and probe design

2 A panel of up to 32 genes should be selected based on your area of interest. Once this has been determined, cDNA sequences of these genes can be used to design 30 nucleotide (nt) antisense probes. To do this we used a custom R script to BLAST 30mer sequences from each transcript against the mouse transcriptome and discarded those with matches of >14 nt to limit off target binding. Then those chose up to 90 probe sequences per gene (dependent on cDNA length). These sequences were then conjugated to binding sites for the bridge-readout probes (complimentary sequences to those found in attachment file), and were flanked by a T7 promoter and PCR primer binding sites, all of which are required for probe synthesis (see next section).

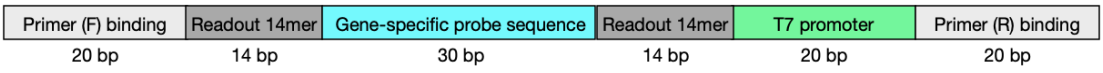


Figure 2: Probe design

An advantage of oligopools as opposed to synthesizing individual probes is that a large number of oligos can be synthesized in a fraction of cost. Furthermore, sub-libraries can be amplified by using different index primers sequences.

## Probe library synthesis

3 Probe library synthesis involves PCR from an oligopool template (in our case synthesized by Twist Bioscience). After purification an in vitro transcription reaction is performed on the amplified template, followed by reverse transcription. Finally, the product is hydrolyzed to fragment the RNA strand, allowing the ssDNA to be purified by ethanol precipitated.

### 3.1 **PCR amplification and product purification**

1. Set up the following PCR reaction

| | A | B | C |
|---|---|---|---|
| | Reagent | 1x volume (ul) | 25x volume (ul) |
| | Water | 36.5 | 912.5 |
| | 5x buffer (HiFi Fidelity with Mg) | 10 | 250 |
| | dNTPs (10mM each) | 1.5 | 37.5 |
| | Forward primer (100uM) | 0.6 | 15 |
| | Reverse primer (100uM) | 0.6 | 15 |
| | DNA template (40pg/ul) | 0.5 | 12.5 |
| | Kapa HiFi Polymerase | 0.5 | 12.5 |

2. Put into a PCR strip/plate, loading 50ul per well.
3. Run the following thermal cycle program:
- 98C for 3 min
- Then 22 cycles of...
- 98C for 5s
- 72C for 30s
- Followed by...
- 72C for 5 min
- 12C hold
4. Pool samples into a 15ml falcon tube (total ~1.2ml)
5. Put 4x DNA purification columns into vacuum manifold *turned on) and add equal volumes of PCR product to each.
6. Wash by adding 750ul Omega wash buffer (with ethanol already added) two times to each column.
7. Place columns in waste collection tube and spin in benchtop microcentrifuge for 1 min at max. speed (~16,000g).
8. Place columns into new eppendorf tubes and add 50ul clean water.
9. Wait for 1 min then centrifuge at full speed for 1 min.
10. Pool eluates and measure concentration.

Typical concentrations are 50-200 ng/ul, therefore ~10-40ug total.

## 3.2 In Vitro Transcription

Assuming a yield of 20ug from the previous step, this would allow 10x transcription reactions to be set up with maximum 2ug per reaction.

1. Set up the following transcription reaction. Note that we used T7 transcriptase produced in-house. However the NEB HiScribe T7 High Yield RNA Synthesis Kit can also be used, according

to the manufacturer's protocol. The 10x volume can be pooled into one tube.

| | A | B | C |
|---|---|---|---|
| | Reagent | 1x volume (ul) | 10x volume (ul) |
| | 10x transcription buffer | 2 | 20 |
| | MgCl2 | 1.2 | 12 |
| | NTPs | 8 | 80 |
| | T7 transcriptase enzyme mix | 2 | 20 |
| | Template (250ng/ul) | 8 | 80 |

2. Incubate at 37C for 15-16h

### 3.3 Reverse Transcription (RT)

1. Set up 2x RT reactions for every T7 reaction (again, these can be pooled) in the following way. Total reaction volume in this case is 560ul.

| | A | B | C |
|---|---|---|---|
| | Reagent | 1x volume (ul) | 20x volume (ul) |
| | dNTPs (10mM each) | 4 | 80 |
| | 5x RT buffer | 4.9 | 98 |
| | Water | 1 | 20 |
| | RT primer (100uM) | 6 | 120 |
| | Rnase inhibitor | 1 | 20 |
| | RTase | 0.5 | 10 |
| | RNA template from transcription reaction | 10.6 | 212 |

2. Incubate at 50C for 1h, then place on ice.

A note on the RT primers: reverse transcription primers were designed to include RNA bases e.g. 5'-GCGTTGrCrGTGCTAArCTCGGArU-3'. This allows hyrolysis of this incorprated sequence during the next step, shortening the final product by 20 bases with the aim of improving probe penetration into the tissue during the hybridization process.

### 3.4 Hydrolysis and precipitation

1. Prepare hydrolysis solution (equal parts 1M NaOH and 0.5M EDTA).
2. Add one reaction volume (560ul) of ‘hydrolysis solution’ to the RT reaction.
3. Incubate at 95C for 10 min.
4. Place on ice and add an equal reaction volume (560ul) of 2M Tris pH7.0.
5. Add 560ul H2O. Total volume is therefore 2.24ml.
6. Add 0.5x volume of 7.5M ammonium acetate (1.12ml).
7. Add 2.5x volume of pre-chilled ethanol (5.6ml).

8. Place in -80C for minimum 2.5h, but can be left for longer term storage.
9. Pre-cool refrigerated centrifuge to 4C.
10. Aliquot solution into 1.5ml Eppendorf tubes (in this case the total volume is ~9ml, so 6 eppendorf tubes are required.
11. Spin at 4C max speed (16,000g) for 30 min.
12. Carefully remove supernatant making sure not to lose white pellet.
13. Add 500ul 80% EtOH and spin again for 5 min.
14. Remove supernatant fully and let pellet dry.
15. Resuspend in 1xTE, let dissolve for ~30min, pool into one tube and measure concentration.

The expected size (58 bases) of the product should be confirmed by polyacrylamide gel electrophoresis. The molarity should be calculated based on how many genes are in the library and an expected molecular weight of 18,850 per oligo (58 bases x 325 Da per base).

## Bridge-readout and amplifier sequences

4 Our bridge-readout and amplifier sequences were tested for orthogonality and signal strength. We have provided the sequences in the attachment which should be ordered as oligonucleotides from DNA synthesis companies and diluted in water or 1xTE to a stock concentration of 100uM. Readout probes need to be conjugated with fluorescent molecule of choice (we use AlexaFluor-488, Cyanine-3, Cyanine-5 conjugated probes).

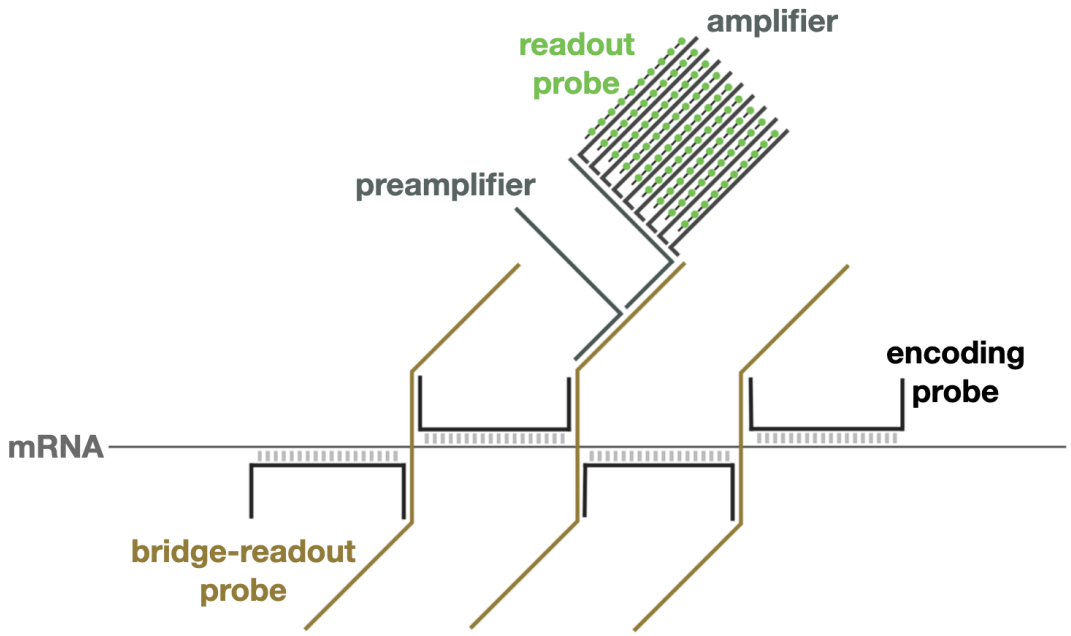


Figure 3: hamFISH branched DNA amplification scheme

One advantage of using hamFISH bridge-readout probes to detect signal is that one can select a sub-selection of genes within the oligopool library to develop by omitting their inclusion.

## hamFISH protocol

5 The following workflow contains information on how to prepare tissue and hybridize hamFISH probes to the sample. We used non-fixed mouse brains that had been submerged immediately in OCT compound following dissection then frozen in dry ice before transfer to -80C for long-term storage.

### 5.1 Coverslip preparation

1. Dilute 50 uL of 3-(trimethoxysilyl)propyl methacrylate in 10 mL of 100% ethanol
2. Add 300 uL of dilute acetic acid (1:10 glacial acetic acid:water)
3. Pour solution onto coverslips and allow to react for 3 minutes
4. Aspirate excess, rinse plates with 100% ethanol twice, and leave to dry
5. Use poly-l-lysine solution used to treat coverslips for 5 min, before aspirating and leave to dry

### 5.2 Tissue preparation and hybridization

1. Cryosection at 10um onto treated coverglass
2. Allow 30 sec to dry then place inside cryostat to freeze until required
3. Fix with ~1mL fixation solution for 10 min
4. Was twice with PBS, 2 min each

5. Transfer coverglass to -20C pre-chilled 100% ethanol, immediately put into -80C for 30 min
6. Remove from -80C and dry at room temp
7. Re-hydrate with ~1 mL 2xSSC for 5 min
8. Aspirate and replace with ~1mL 4%SDS for 5 min on rotating platform
9. Wash three times quickly in 2xSSC, followed by a 3 min wash in 2xSSC
10. Wash 2x 1 min in 1xTE
11. Wash three times quickly in 2xSSC, followed by a 3 min wash in 2xSSC
12. Aspirate and cover with ~1mL Protease solution (1:30000 subtilisin in 1x PBS) for 7.5min
13. Wash three times quickly in 2xSSC, followed by a 3 min wash in 2xSSC
14. Transfer coverglass to humidified chamber and wash with pre-hyb buffer for 3 min
15. Prepare Hyb buffer with 0.1uM probe library
15. Aspirate pre-hyb solution and replace with enough Hyb buffer to cover sample
16. Cover with small square of parafilm, seal in ziplock bag and put into 37C oven

## 5.3 Amplification

1. Wash three times quickly with 0.2xSSC, followed by ~5min 0.2xSSC wash at room temp
2. Wash with 0.2xSSC again and incubate for 1h in 37C oven
3. Prepare 50ul amplification solutions (10nM anchor, preamp and amp oligos in Amplification buffer)
4. Incubate sample with anchor solution for 30 min at 37C under parafilm square
5. 2x 10 min washes in amplifier wash buffer, followed by a 1 min 2xSSC wash
6. Incubate sample with preamp solution for 30 min at 37C under parafilm square
7. 2x 10 min washes in amplifier wash buffer, followed by a 1 min 2xSSC wash
8. Incubate sample with amp solution for 30 min at 37C under parafilm square
9. 2x 10 min washes in amplifier wash buffer, followed by a 1 min 2xSSC wash

## 5.4 Gel embedding and signal development

1. Dilute 500uL of 2mM acrylic acid NHS ester (in DMSO) in 4.5mL PBS
2. Cover coverslip in acrylic acid solution for 2h
3. Exchange to monomer buffer for 30 min
4. Aspirate solution off coverslips
5. Prepare polymerisation mix - 250ul monomer buffer + 0.5ul TEMED + 5ul 10% APS
6. Place 25ul polymerisation mix on GelSlick glass plate
7. Place overturned coverslip of polymerisation mix
8. Leave for 30min at room temp
9. Wash in 2xSSC to remove coverslip
10. Incubate in digest buffer on rotating platform for 1h
11. Wash three times quickly in 2xSSC, followed by a 3 min wash in 2xSSC
12. Prepare labelled probe solution (50nM of each labelled probe oligo in amplification hyb buffer)
13. Incubate sample with labelled probe solution for 10 min at 37C under parafilm square
14. Quick wash with 2xSSC followed by 3 min wash in DAPI solution

15. 2x 3min washes in 2xSSC
16. Image

## Sequential imaging

6 Sequential round of signal development and imaging are required to readout transcript signal from all probes. In each signal development round, the sample must undergo another hybridization step with buffer containing toehold probes to competitively remove readout probes from the previous round, as well as readout probes for the next round of imaging. Below is a table that demonstrates an example of buffer compositions for a typical hamFISH experiment.

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| | Hybridization round | AlexaFluor-488 readout probe | Cyanine-3 readout probe | Cyanine-5 readout probe | 1st toehold probe | 2nd toehold probe | 3rd toehold probe |
| | 1 | 1 | 2 | 3 | - | - | - |
| | 2 | 5 | 6 | 7 | 1 | 2 | 3 |
| | 3 | 8 | 9 | 10 | 4 | 5 | 6 |
| | 4 | 11 | 12 | 13 | 7 | 8 | 9 |
| | 5 | 14 | 15 | 16 | 10 | 11 | 12 |
| | 6 | 17 | 18 | 19 | 13 | 14 | 15 |
| | 7 | 20 | 21 | 22 | 16 | 17 | 18 |
| | 8 | 23 | 24 | 25 | 19 | 20 | 21 |
| | 9 | 26 | 27 | 28 | 22 | 23 | 24 |
| | 10 | 29 | 30 | 31 | 25 | 26 | 27 |
| | 11 | 32 | - | - | 28 | 29 | 30 |

Note that bit order can be modified as well as conjugation of different fluorescent tags. This may be needed if channels suffer from bleedthrough and therefore highly expressed genes may need to be developed in channels that do not bleedthrough to others.

Samples were imaged on a custom-built imaging platform based with 405, 488, 568 and 647 nm solid-state lasers (OBIS, Coherent). The base of the microscope was Nikon Ti-U (Nikon), equipped with a custom FocusLock system. The coverslip was assembled in a flow chamber (Bioptechs, FCS2) attached to a home-built fluidics system that controlled hybridization flow through the sample. The fluidics system was comprised of a peristaltic pump (Gilson, MINIPULS 3) and three eight-way valves
(Hamilton, MVP RS-232 valves), all computer-controlled by HAL/Dave and Kilroy software (https://github.com/ZhuangLab/storm-control/blob/master/storm_control/README.md) ensuring fully automated control of the whole system.

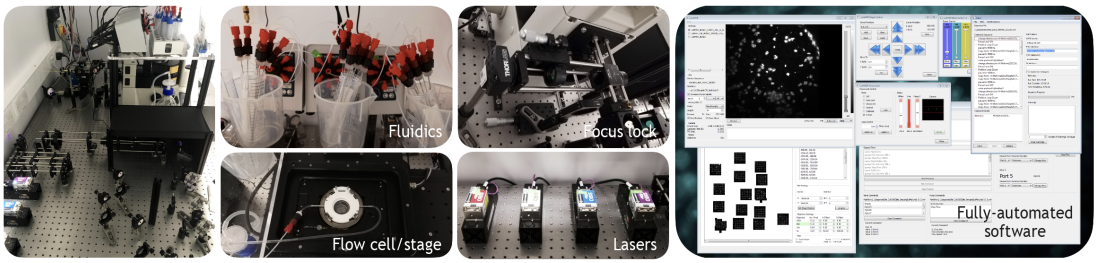


Figure 4: Microscopy setup used for hamFISH