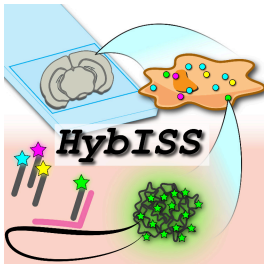
Feb 04, 2020

HybISS: Hybridization-based In Situ Sequencing



Nucleic Acids Research

In 1 collection

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Daniel Gyllborg[1], Mats Nilsson[1]

[1]Department of Biochemistry and Biophysics, Science for Life Laboratory, Stockholm University, Solna, Sweden

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Daniel Gyllborg
Stockholm University

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Hybridization-based In Situ Sequencing (HybISS): spatial transcriptomic detection in human and mouse brain tissue

Daniel Gyllborg, Christoffer Mattsson Langseth, Xiaoyan Qian, Sergio Marco Salas, Markus M. Hilscher, Ed S. Lein, Mats Nilsson
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We use this protocol and it's working

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Abstract

Protocol for multiplexed in situ sequencing in tissue sections as an image-based spatial transcriptomic method. This is the second iteration of In Situ Sequencing (HybISS: Hybridization based In Situ Sequencing) based on the principles published in Ke et al. Nature Methods, 2013 and more recently Qian et al. Nature Methods, 2019. Here we present a modified method in detection chemistry using sequencing by hybridization approach for combinatorial decoding. This results in a more robust method for detecting larger probe panels for a more high-throughput in situ sequencing method.

Guidelines

This protocol has been thoroughly tested on mouse and human brain sections. Other tissues of origin have also been tested and work as well, some requiring minor adjustments and optimization for certain conditions such as pretreatments and fixations.

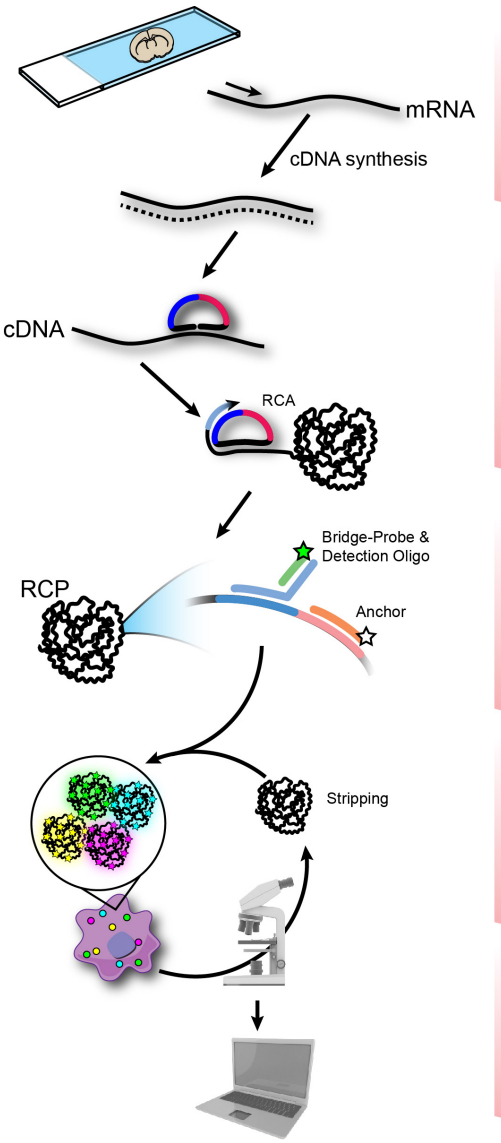
In addition to the cited and linked bioRxiv preprint, see also the following publications for additional references and information on in situ sequencing, padlock probes and rolling circle amplification:

Probabilistic Cell Typing Enables Fine Mapping of Closely Related Cell Types in Situ
Qian X, Harris KD, Hauling T, Nicoloutsopoulos D, Muñoz-Manchado AB, Skene N, Hjerling-Leffler J, Nilsson M
Nat Methods. 2020 Jan;17(1):101-106.
doi: 10.1038/s41592-019-0631-4

In Situ Sequencing for RNA Analysis in Preserved Tissue and Cells
Ke R, Mignardi M, Pacureanu A, Svedlund J, Botling J, Wälby C, Nilsson M.
Nat Methods. 2013 Sep;10(9):857-60.
doi: 10.1038/nmeth.2563

Spatial and temporal localization of immune transcripts defines hallmarks and diversity in the tuberculosis granuloma
Carow B, Hauling T, Qian X, Kramnik I, Nilsson M, Rottenberg ME.
Nat Commun. 2019;10(1):1823.
doi:10.1038/s41467-019-09816-4

Protocol Workflow Overview



Day 1:
Tissue preperation and reverse transcription
Steps # 1-15

Day 2:
Padlock probe hybridization and ligation
Steps # 16-20

Rolling circle amplification (RCA)
Steps # 21-25

Day 3:
Bridge-probe hybridization
Steps # 26-34

Detection oligo hybridization
Steps # 35-39

Day 4:
Imaging
Steps # 40-41

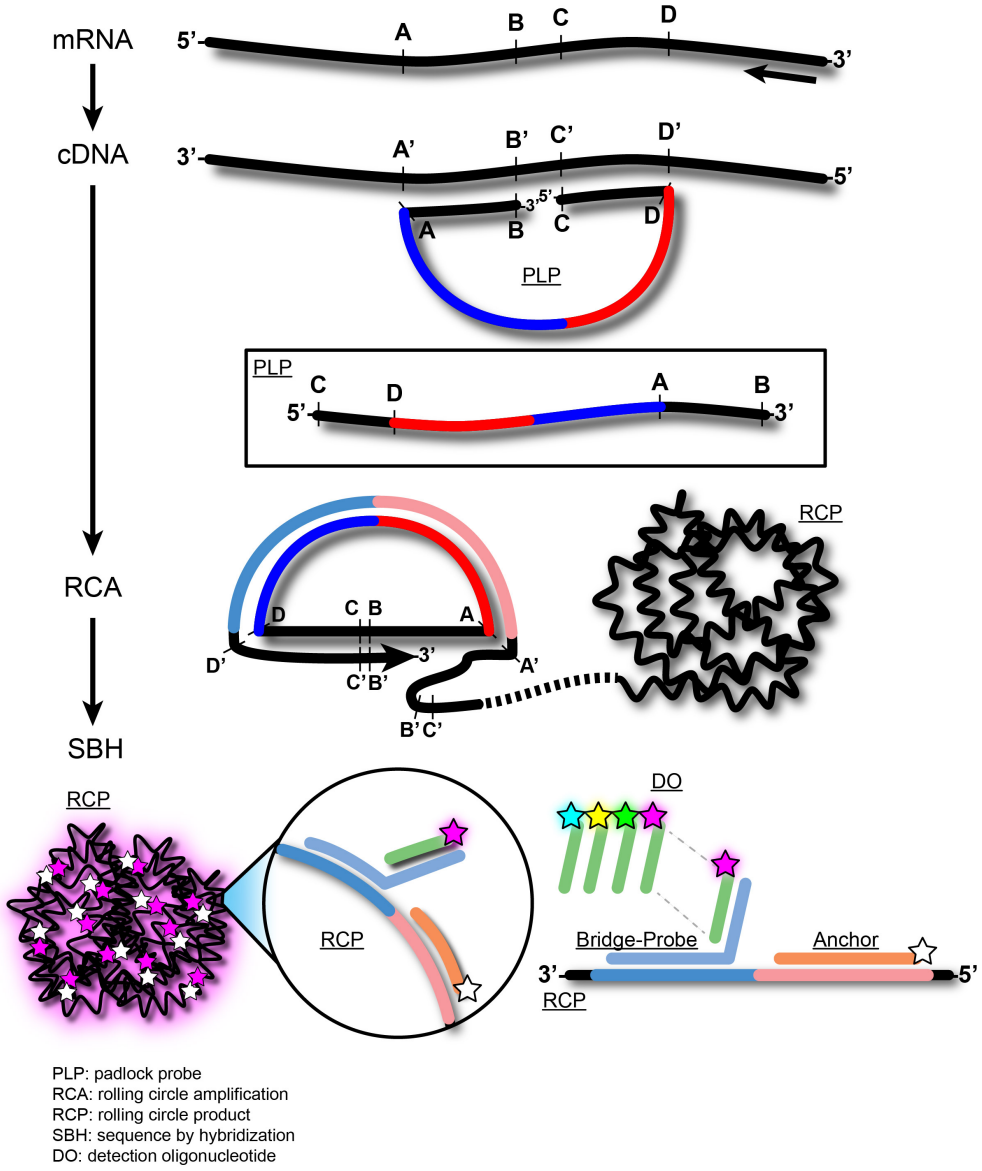
Stripping of Bridge-probe and detection oligo
Steps # 42-49

Day 5+:
Repeated Bridge-probe hybridization, detection oligo hybridization, imaging and stripping for additional cycles
Steps # 50-53

Overview of the general workflow of the protocol. Depending on incubation times, the days stated are approximations. For example, imaging can already start on day 3 since there are no overnight steps after RCA.

Padlock Probe (PLP) Design

Design and selection of target sequences can be done by following an in-house pipeline: **GitHub Padlock Design**. This will give you target sequences for both arms of the padlocks. Below we show the suggested design of the padlock probe. For more detailed insight, we refer you to the accompanying preprint publication.



Visualization of how PLP works and direction of synthesis and design considerations.

Equipment

- Hydrophobic pen
- Forceps
- 30°C, 37°C, and 45°C incubators
- Humidity chamber for slide incubation
- SecureSeal hybridization chambers (Grace Bio-Labs)
- Coverslips
- Coplin jars or similar for washing of slides
- Adhesive microscopy slides (e.g., Menzel Gläser SuperFrost Plus)

- Wide-field epifluorescence microscope (6-channel)
- Plate rocker

General Guideline and Controls

1. This protocol has been optimized for fresh frozen mouse brain sections as well as human brain tissue. However, other tissues have been shown to work robustly with this protocol as well. Optimization for specific tissues may be required such as fixation, pretreatment and incubation conditions.
2. Enzymes and other reagents included in this protocol can be purchased from several different manufacturers (e.g. New England Biolabs or Thermo Fisher Scientific) and have performed equally well in our hands. Optimization, testing, and benchmarking should be performed whenever new reagents from different vendors are used.
3. Stock concentrations of reagents could vary depending on vendor used. Adjust tables so that final concentration of reagents is the same. Optimization might also be required.
4. This protocol involves RNA work and special care needs to be taken to prevent RNases. It is recommended to have designated space and equipment for RNA work and should be treated/cleaned with commercially available RNase and DNAse inactivating agents and then wiping with 100% ethanol after treatment.
5. Using sterile, disposable, RNase-free plasticware (pipette tips, slide boxes, tubes, and flasks) is recommended.
6. PBS and water can be purchased as RNase free from numerous vendors. It is also possible to treat PBS and water with DEPC (diethyl pyrocarbonate) to make them RNase free.
7. Synthetic DNA targets can be used to validate specificity of padlock probes. Rolling circle amplification (RCA) can be monitored in vitro by staining rolling circle products (RCPs) with either intercalating dyes (SYBR dyes) or decorator probes and visualized under a microscope or qPCR system.
8. This protocol assumes correct design of padlock probes (PLPs), Bridge-probes, and detection oligos (DOs) for sequencing. See publications for further details on probe design to target genes of interest.
9. This protocol does not go into detail on padlock probe design and analysis of data. See publications for further detail and image analysis.
10. Unlike previous in situ sequencing protocols from our lab, this protocol avoids the use of ethanol dehydration steps, and minimal detergent use in wash buffers. Additionally, do not let section dry out between steps. Let sections remain in washing solutions while preparing the next steps.

Abbreviations

PLP: padlock probe
RCA: rolling circle amplification
RCP: rolling circle product
SBH: sequence by hybridization
DO: detection oligonucleotide

Materials

MATERIALS

- BSA-Molecular Biology Grade - 12 mg **New England Biolabs** **Catalog #**B9000S
- Nuclease-Free Water
- TrueBlack™ Lipofuscin Autofluorescence Quencher **Gold Biotechnology** **Catalog #**TB-250
- TRANSCRIPTME Reverse Transcriptase **BLIRT** **Catalog #**RT32
- RNase H **BLIRT** **Catalog #**RT34
- Exonuclease I (20 U/µL) **Thermo Scientific** **Catalog #**EN0582
- SlowFade™ Gold Antifade Mountant **Invitrogen - Thermo Fisher** **Catalog #**S36936
- Superfrost Plus™ Adhesion Microscope Slides **Thermo Scientific** **Catalog #**J1800AMNT
- RIBOPROTECT Hu RNase Inhibitor **BLIRT** **Catalog #**RT35
- Phi-29 DNA Polymerase **Monserate Biotech** **Catalog #**4002
- Tth DNA Ligase **BLIRT** **Catalog #**EN13
- dNTPs mix **BLIRT** **Catalog #**RP65
- Formamide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**F9037
- Formaldehyde solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**252549
- Hydrochloric acid (HCl) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**258148
- Glycerol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**G5516
- DAPI **Biotium** **Catalog #**40043
- PBS

Other common solutions needed to make buffers, etc.:

Tris-HCl (pH 8.3)
Tris-HCl (pH 7.5)
KCl
MgCl2
NaCl
DTT
NAD
Triton X-100
EDTA
(NH4)2SO4
20X SSC
Sodium phosphate
ATP

Troubleshooting

Safety warnings

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 and solutions containing reagents mixed in.

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 repeatedexposure 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.
Suspected of causing genetic defects.
Causes damage to organs (Eyes).

TrueBlack Lipofuscin Autofluorescence Quencher (TLAQ):
Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution and dispose of waste appropriately.
Harmful in contact with skin.
Causes eye irritation.
Harmful if inhaled.
May damage the unborn child.

Before start

This working protocol has been setup and optimized to work on fresh frozen human and mouse brain tissue. Depending on the species origin of the tissue, certain steps/sections in this protocol require different handling of tissue and is indicated in section header. For example, human brain sections requires additional treatment for lipofuscin quenching.

Other tissue and other species might require different pretreatment conditions that would need to be optimized.

Enzyme Buffer solutions:
Enzyme buffer solutions can be prepared and aliquoted prior to experiment and stored at -20°C.
Enzyme buffers are often provided along with enzyme from manufacturer and can be used to equal effectivenesss. Here we provide typical/similar composition of those buffers that can be made yourself.

10X Reverse Transcriptase Buffer:
- 500 mM Tris-HCl (pH 8.3)
- 750 mM KCl
- 30 mM $MgCl_2$
- 100 mM DTT

10X Tth DNA Ligase Buffer:
- 200 mM Tris-HCl (pH 8.3)
- 250 mM KCl
- 100 mM $MgCl_2$
- 5 mM NAD
- 0.1% Triton X-100

10X Phi29 Buffer:
- 500 mM Tris-HCl (pH 8.3)
- 100 mM $MgCl_2$
- 100 mM $(NH_4)_2$ SO_4

Other Buffers and solutions:

Nuclease-free Water (NF-H_2O)

2X Hybridization Buffer (made fresh on day of use):
- 4X SSC
- 40% Formamide
- NF-H_2O

20X SSC (saline-sodium citrate) buffer
- (Purchased as RNase-free from vendor)

Phosphate buffered saline (1X PBS)
- (Purchased as RNase-free from vendor)

PBS-Tween (PBS-T)
- 1X PBS
- 0.05% Tween-20

Tissue Sample Preparation: Mouse Section

1 !!! Note: For human sections, proceed to **Step 4** !!!

Fresh frozen tissue samples are embedded in OCT (optimal cutting temperature) compound and stored at -80°C until cryosectioning.

Tissue is cryosectioned at 10 µm thickness and collected on SuperFrost Plus adhesion slides and can be stored at -80°C until used for experiment.

Note

Cryosections thickness of 5-20 µm have been tested. We typically cryosection at a thickness of 10 µm for optimal results.

2 Slides with sections are taken from -80°C and left at room temperature (RT) for 3-5 min to air dry.

00:03:00 Thaw at RT

3 Fixation performed with freshly prepared 3% formaldehyde in PBS for 5 min at RT.

Formaldehyde solution is applied directly on top of the section on flat laying slide.

00:05:00 Tissue fixation

Safety information

Safety precaution: Formaldehyde

Proceed to **Step 7**.

Tissue Sample Preparation: Human Section

4 Fresh frozen tissue samples are embedded in OCT (optimal cutting temperature) compound and stored at -80°C until cryosectioning.

Tissue is cryosectioned at 10 µm thickness and collected on SuperFrost Plus adhesion slides and can be stored at -80°C until used for experiment.

Safety information

Extra precautions should be taken with tissue of human origin. Obey local guidelines/rules and take appropriate pecautionary measures when handling tissue, especially fresh tissue to avoid any risks.

5 Slides with sections are taken from -80°C and left at room temperature (RT) for 3-5 min to air dry.

00:03:00 Thaw at RT

6 Fixation performed with freshly prepared 3% formaldehyde in PBS for 30 min at RT.

Formaldehyde solution is applied directly on top of the section on flat laying slide.

00:30:00 Tissue fixation

Safety information

Safety precaution: Formaldehyde

Tissue Sample Preparation: Mouse/Human Section

7 Formaldehyde on section is discarded and tissue section is washed with PBS at RT.

Note

To discard solutions from slides, tilt slide to allow excess liquid to pour off into disposal container. Additional liquid can be removed by tapping long edge of slide against a paper towel.

For all washing steps, use a reasonable amount of washing buffer on section. For a mouse coronal section, 200 µl is sufficient.

8 Section washed two more times with PBS.

9 The tissue is permeabilized with 0.1 M HCl (in H_2O) at RT for 5 min.

Note

This can be performed by submerging glass slide in ~20 ml solution of 0.1 M HCl, in a jar or container that can hold slides.

00:05:00 HCl permeabilization

Safety information

Safety precaution: Hydrochloric acid

Note

Permeabilization of tissue needs to be optimized. Additional pre-treatments are possible such as incubation with Pepsin. Permeabilization should be optimized depending on the source of the tissue.

10 After permeabilization, tissue sections are washed with PBS two times.

11 Sections are then ethanol dehydrated in order to aid thin the adhesion of SecureSeal chambers.

Submerge sections/slides in 70% Ethanol for 1min.
Transfer and submerge in ~100% Ethanol for 1min.

Remove slide and let air dry.

Note that it is also possible to mount SecureSeal chambers without dehydrating slides, you just have to ensure a dry surface area around tissue for proper adhesion of the chamber.

12 With a dry surface around tissue section, apply appropriate size SecureSeal hybridization chambers over tissue.

Note

SecureSeal hybridization chambers come in different sizes, shapes, and depths. Small chambers have a volume of ~50 µl (round, 9 mm diameter, and 0.8 mm deep: enough for half a coronal section of a mouse brain). For larger tissue specimens, larger chambers and shapes can be used and consequently volumes in protocol should be adjusted.

Chambers have to be mounted on dry surfaces for a proper seal to prevent reagent content loss during incubations.

13 Sections and chamber are washed by applying PBS-T to chamber inlet and then followed by one wash of PBS. Let PBS remain in chamber until the next steps' reagents are prepared.

Note

Apply and remove solutions to chamber by tilting the slide/chamber slightly and pipetting into lower inlet. This will aid in preventing bubble formation within the chamber.

In Situ Reverse Transcription

14 Reagents for reverse transcription are combined as in the table below.

Note

All following tables in this protocol have been calculated to a final volume of 50 µl and should be adjusted accordingly.

	Reagent	[Stock]	[Final]	1x (µl)
	NF-H_2O			34.75
	Reverse Transcriptase Buffer	10X	1X	5
	dNTPs	25 mM	500 µM	1
	BSA	20 µg/µl	0.2 µg/µl	0.5
	Random Decamers	100 µM	5 µM	2.5
	RNase Inhibitor (Riboprotect)	40 U/µl	1 U/µl	1.25

	Reverse Transcriptase (TranscriptME)	200 U/µl	20 U/µl	5
	Total			50

Note: H_2O is used to get total volume required. Other vendors can supply reagents at various stock concentrations and adjustments need to be made accordingly, including possible optimization of concentrations.

15 PBS is removed from SecureSeal chamber and combined reagents from table above added.
Chamber inlets are sealed and slide placed in a humidity chamber.

Note

Any container to hold slides in place, lying flat and allow moisture retention will suffice. We use small slide boxes with wet (Milli-Q water) whatman paper in bottom of container.

Slide and humidity chamber incubated from 6 hours to overnight at 37°C.

37 °C Reverse transcription incubation

Padlock Probe Hybridization and Ligation

16 The next day, samples are taken out of incubator.

Reverse transcription reagents are carefully removed and postfixation is performed with 3% formaldehyde in PBS for 40 min at RT.
Note that there is no wash step in between.

00:40:00 Postfixation

Safety information

Safety precaution: Formaldehyde

17 Remove PFA and wash chambers 2 times with PBS. Let PBS remain in chamber until the next steps' reagents are prepared.

18 Combine reagents for PLP hybridizations in table below.

	Reagent	[Stock]	[Final]	1x (µl)
	NF-H_2O			22
	Ligase (Tth) buffer	10X	1X	5
	KCl	1M	50 mM	2.5
	Formamide	100%	20%	10
	Padlock Probe(s)	0.5 µM	10 nM each	1
	BSA	20 µg/µl	0.2 µg/µl	0.5
	Tth DNA Ligase	5 U/µl	0.5 U/µl	5
	RNaseH	5 U/µl	0.4 U/µl	4
	Total			**50**

Note: H_2O is used to get total volume required. Other vendors can supply reagents at various stock concentrations and adjustments need to be made accordingly, including possible optimization of concentrations.

Note

The amount of padlock probes will vary depending on user/experiment and initial concentration may vary. Final concentration of each padlock probe should be around 10 nM each. This may require optimization depending on the total amount of padlock probe targets.

Safety information

Safety precautions: Formamide

19 Remove PBS from chambers and add combined reagents from above table to chamber.

Seal chamber inlets and place slide in humidity chamber.
Incubate at 37ºC for 30 min.

37 °C

00:30:00 PLP incubation (1)

20 After 30 min incubation, transfer humidity chamber with slide to 45ºC incubator to incubate for an additional 1 hour 30 min.

45 °C

01:30:00 PLP incubation (2)

Rolling Circle Amplification (RCA)

21 Remove reagents from chamber after incubation.

Wash chamber 2 times with PBS.

Let PBS remain in chamber until next reagents are combined.

22 Reagents for RCA are combined as in the table below.

	Reagent	[Stock]	[Final]	1x (µl)
	NF-H_2O			33
	Phi29 buffer	10X	1X	5
	Glycerol	50%	5%	5
	dNTPs	25 mM	0.25 mM	0.5
	BSA	20 µg/µl	0.2 µg/µl	0.5
	Exonuclease I	20 U/µl	0.4 U/µl	1
	Phi29 polymerase	10 U/µl	1 U/µl	5
	Total			**50**

Note: H_2O is used to get total volume required. Other vendors can supply reagents at various stock concentrations and adjustments need to be made accordingly, including possible optimization of concentrations.

23 PBS from chamber is removed and combined reagents from table above are applied to chamber.

24 Seal chamber inlets and place slide in humidity chamber and incubate at 37°C for up to 4 hours and then switch to 30°C overnight.

37 °C Incubation

04:00:00

30 °C Overnight incubation

Note

It is also possible to place directly at 30°C overnight if there are time constraints. Exonuclease I has an optimal working temperature at 37°C and phi29 polymerase at 30°C.

25 After overnight incubation, remove reagents from chamber.

Wash chamber twice with PBS. Let PBS remain until next step.

Autofluorescence Quenching: Human Tissue

26

Note

For human tissue or tissue that is know to give high autofluorescence, it is recommended to treat the tissue at this point with some sort of autofluorescent quencher. Here we use TrueBlack Lipofuscin Autofluorescent Quencher. Other quenchers can also be tested and optimized.

If quenching is not needed, Steps #26-28 can be skipped.

Combine reagents for TrueBlack Autofluorescence Quencher (TLAQ) as per manufacture's instructions.

- 20X TLAQ stock diluted in 70% EtOH to a working 1X TLAQ solution.

Safety information

Safety precaution: TLAQ

27 Remove PBS and apply TLAQ solution to chamber.

Incubate at RT for 30 sec to 1 min.

00:00:30 TLAQ

Note

Various tissue sources could require different TLAQ incubation times that would need to be tested. For brain tissue 30sec - 2min has been tested and works well.

Here, TLAQ is applied after RCA and before detection oligo probe hybridization. It is also possible to perform TLAQ treatment after detection oligo hybridization.

28 Remove TLAQ solution and wash immediately with PBS.

Repeat PBS wash 2 more times (3 total).
Let PBS remain until next step.

Note

When TLAQ has been applied, detergents and ethanol will wash out TLAQ and dilute its effects on autofluoresence. It is important to avoid them from this point on. High concentrations of formamide can also bleach out effect of TLAQ. TLAQ can be reapplied again if necessary. TLAq treated sections should also be kept in the dark as much as possible as it is light sensitive.

Sequence By Hybridization: Bridge-Probe and Detection Oligo

29 After washing steps, carefully remove SecureSeal chamber.

Note

Remove SecureSeal chamber with the aid of forceps.

Then wash section one more time with PBS.

30 Then carefully remove as much liquid as possible from area surrounding tissue section. Use paper towel to dry area if needed. Then, with a hydrophobic pen, draw a barrier surrounding tissue section.

After barrier has been drawn around tissue and dried slightly, apply additional PBS to section to prevent it from drying out and proceed to next step.

Note

We use ImmEdge Hydrophobic Barrier PAP Pen by Vector Laboratories (Cat. No. H-4000). Other hydrophobic pens have shown to impede the in situ sequencing visualiztion.

It is also possible to reapply SecureSeal hybridization chambers instead of using hydrophobic pen for subsequent hybridization steps. We find it easier to continue all steps without the use of the chamber.

Note that the same volume should be used on tissue sections as what the chamber volume was.

31 Combine reagents for Bridge-Probe hybridization as in table below.

	Reagent	**[Stock]**	**[Final]**	**1x (µl)**
	NF-H_2O			24.5
	2X Hybridization buffer	4XSSC, 40% Formamide	2XSSC, 20% Formamide	25
	Bridge-Probes	10 µM	0.1 µM	0.5
	Total			**50**

Note: H_2O is used to get total volume required.

Final concentration of Bridge-Probes should be optimized but a range between 10-100 nM is ideal, this could depend on the number of targets/padlock probes added.

32 PBS is removed from section and is washed once with 2XSSC. Reagents are combined from table above and are applied to section.

33 Incubate Bridge-probe reagents for 1 hour at RT in dark, on rocker.

01:00:00 Bridge-Probe incubation

Note

All hybridizations (Bridge-Probe and detection oligos) at room temperature are performed on a slow, 360°, low angle rotation rocker to ensure distribution of reagents evenly on tissue sections.

34 Discard reagents and wash 2 times with 2XSSC.
Let 2XSSC remain until next step reagents are prepared.

35 Combine reagents for dection oligo hybridization as in table below.

	Reagent	**[Stock]**	**[Final]**	**1x (µl)**
	NF-H_2O			22.75
	2X Hybridization buffer	4XSSC, 40% Formamide	2XSSC, 20% Formamide	25
	DO_Seq_1	10 µM	0.1 µM	0.5
	DO_Seq_2	10 µM	0.1 µM	0.5
	DO_Seq_3	10 µM	0.1 µM	0.5
	DO_Seq_4	10 µM	0.1 µM	0.5
	Anchor (see note)			
	DAPI	100 µg/µl	0.5 µg/µl	**0.25**
	Total			**50**

Note: H_2O is used to get total volume required. Various stock concentrations are possible and adjustments need to be made accordingly, including possible optimization of concentrations.

Note

Anchor: If anchor is used, the same final concentration as detection oligos should be used. It is possible to work without an anchor when running image analysis. Anchor imaging is typically used for spot detection and alignment across cycles.

36 2XSSC removed and combined reagents from table added to chamber.

37 Slides are incubated in dark at RT on rocker for 1 hour.

01:00:00 DO incubation

38 After incubation, section is washed with PBS 2 times.

39 Remove as much PBS as possible and then add small drop of mounting media and apply cover slip to slide section.

Note

We use SlowFade Gold Antifade Mountant as it has shown to perform well with our conditions and also allows for removal of cover slip in future steps.

10 µl of mounting media should be enough for the area of one mouse brain coronal section.

Note

Slides at this point can be stored at +4°C with mounted coverslips in the dark for several weeks. It is recommended to image sections soon after detection oligos are added.

Imaging

40 Section can now be imaged.

Note

Labs will have different microscope set ups and identical setups are not needed. Fluorophore dyes can be interchanged depending on your own microscopes capabilities. The number of detection oligo sequences presented here is 4, but this is only limited by your microscopes ability to distinguish fluorophores and can be adjusted to be more or less which would also require adjustments to your combinatorial decoding scheme.

We use a Zeiss Axio Imager.Z2 epifluorescent widefield microscope. It is equipped with either a metal halide lamp or 6-channel LED light source and sCMOS camera. A good filter setup is essential to provide good wavelength seperation and minimal crosstalk between different channels. A typical imaging run includes DAPI for nuclei, 4 seperate fluorophores for the different detection oligo sequences and one channel for anchor detection.

41 Imaging is set up to do repeated cycles of same region every round. Tile region outlines are set up for the first cycle and saved. After every round/cycle, slides are placed back in stage slide holder to the same position for proper alignment. This is aided with the use of an automatic stage that can calibrate to a reference point every time it is used so tile regions have the same coordinates.

Stripping Bridge-Probes and Detection Oligos

42 Submerge slide with coverslip in clean PBS in a coplin jar and let sit for a few minutes. Proceed to next step during this time and prepare reagents.

43 While slide is sumberged, prepare stripping solution and humidity chamber.

Combine reagents for stripping solution as in table below. Preincubate combined reagents and humidity chamber at 30°C until used in Step 47.

	Reagent	[Stock]	[Final]	1x (µl)
	NF-H_2O			25
	Formamide	100%	65%	65
	SSC	20X	2X	10
	Total			**100**

Note: After incubation of section with stripping solution, section is washed once with additional stripping solution, therefore prepare twice as much needed.

Safety information

Safety precaution: Formamide

Prepare 2XSSC washing solution. For 5 ml: 500 µl 20XSSC + 4.5 ml NF-H_2O.

44 Remove slide from PBS and if cover slip does not slide off by itself, either let it sit longer in PBS or carefully slide it off with the aid of forceps.

Remove excess PBS from slide.

45 Wash section 2 times with PBS to remove residual mounting media

46 Discard PBS and wash tissue section 5 times with 2XSSC

47 Place slide in prewarmed humidity chamber. Apply prewarmed stripping solution on top of section and place in 30°C incubator for 30 min. Place any remaining stripping solution in the incubator as well.

30 °C 00:30:00 Stripping incubation

48 After 30 min, remove solution from section and wash 1 time with remaining stripping solution.

49 Wash 5 times with 2XSSC

Repeated Cycles

50

Now the Bridge-Probe:Detection Oligo has been stripped from the tissue.

From this point, next cycle can be performed as before with next cycle of Bridge-probes.

Note

If next cycle of Bridge-probes is not done immediately, section can be stored with PBS at +4°C for short-term storage. For longer-term storage, mount section with mounting media and a coverslip and store at +4°C, then remove and wash section as before to proceed with next round.

51 Repeat cycle by applying Bridge-probes for next cycle.

Hybridization: Steps 29-39
Imaging: Steps 40-41
Stripping: Steps 42-49

52 Repeat cycles until combinatorial barcodes can be decoded. It is also possible to re-do cycles if problems arise with some rounds.

Image Analysis

53 We refer you to the accompany publication and previous publications from the lab for image analysis. All tools and pipelines are available through our GitHub page.