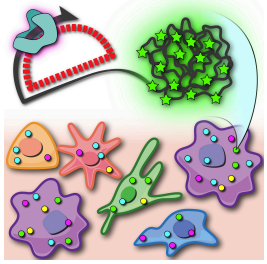
Feb 07, 2019 Version 1

In situ sequencing for RNA analysis in tissue sections V.1



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Daniel Gyllborg[1], Chika Yokota[1], Mats Nilsson[1]

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

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We use this protocol and it's working

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Keywords: in situ sequencing, iss, spatial transcriptomics, padlock probes, sequence by ligation, single cell, rolling circle amplification, RCA, rna analysis in tissue section, analysis of short rna fragment, methodolgy of spatial transcriptomic, rna analysis, spatial transcriptomic, short rna fragment, rna molecules at the single cell level, use of barcode sequencing, barcode sequencing, sequencing method, rna molecule, identification of numerous gene, gene expression, single cell level, identification of cell type, genes of interest, gene, targeted analysis

Abstract

In situ sequencing method for parallel targeted analysis of short RNA fragments in morphologically preserved tissue. This protocol can be used to detect RNA molecules at the single cell level to aid in the identification of cell types according to their gene expression. The technique uses padlock probes to target desired genes of interest and rolling circle amplification to amplify signal for a high throughput methodolgy of spatial transcriptomics. With the use of barcode sequencing, identification of numerous genes is possible through multiplexing.

Attachments

nmeth.2563.pdf
1.5MB

Guidelines

The following protocol is based off of **In situ sequencing for RNA analysis in preserved tissue and cells.** (Ke R et al., Nat. Methods, 2013) with some modifications and focus on fresh frozen tissue sections.

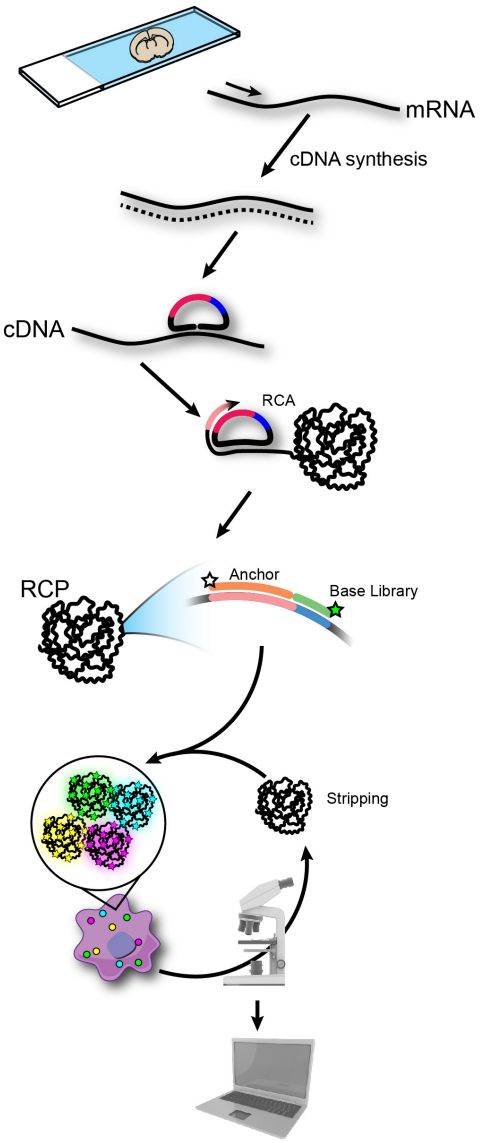
See also following publications for additional references:

Padlock Probes to Detect Single Nucleotide Polymorphisms
Krzywkowski T, Nilsson M.
Methods Mol Biol. 2018;1649:209-229.
doi: 10.1007/978-1-4939-7213-5_14.

In situ detection and genotyping of individual mRNA molecules
Larsson C, Grundberg I, Söderberg O, Nilsson M.
Nat Methods. 2010 May;7(5):395-7.
doi: 10.1038/nmeth.1448.

A spatial atlas of inhibitory cell types in mouse hippocampus
Qian X, Harris KD, Hauling T, Nicoloutsopoulos D, Manchado AM, Skene N, Hjerling-Leffler J, Nilsson M.
bioRxiv 431957;
doi: https://doi.org/10.1101/431957

Protocol Workflow Overview



Day 1:
Tissue preperation and reverse transcription
Steps #**1-11**

Day 2:
Padlock probe hybridization and ligation
Steps #**12-17**

Rolling circle amplification (RCA)
Steps #**18-21**

Day 3:
Anchor primer hybridization
Steps #**22-15**

Sequence by ligation
Steps #**26-32**

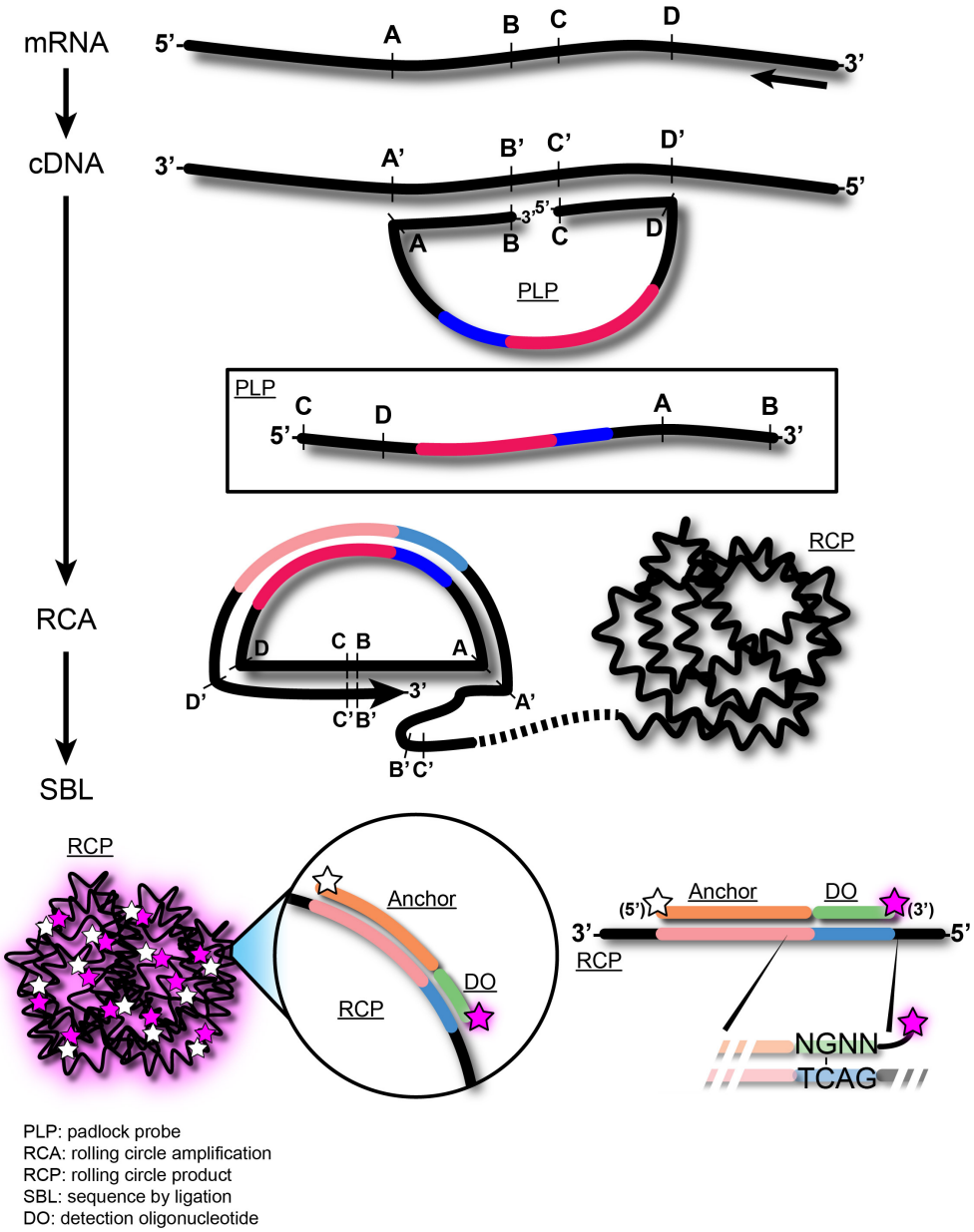
Day 4:
Imaging
Steps #**33-34**

Stripping of anchor and sequencing primer
Steps #**35-44**

Day 5+:
Repeated anchor primer hybridization, sequencing by ligation, imaging and stripping for other bases
Steps #**22-44**

Overview of the general workflow of the protocol. Depending on incubation times, the days stated are approximations.

Padlock Probe (PLP) Design



PLP: padlock probe
RCA: rolling circle amplification
RCP: rolling circle product
SBL: sequence by ligation
DO: detection oligonucleotide

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

Equipment

- Hydrophobic pen (see **Note 1**)
- Forceps
- 30°C, 37°C, and 45°C incubator
- Humidity chamber for slide incubation (see **Note 2**)
- Secure-Seal hybridization chambers (Grace Bio-Labs) (see **Note 3**)
- Coverslips (see **Note 4**)
- Coplin jars or similar for washing of slides

- Adhesive microscopy slides (e.g., Menzel Gläser SuperFrost®).
- Wide-field epifluorescence microscope (6-channel) (see **Note 5**)

General Guidelines and Controls

1. This protocol has been optimized for fresh frozen mouse brain sections. However, other tissues have been shown to work robustly with this protocol as well. Optimization for specific tissues may be required such as fixation and pretreatment conditions.
2. Enzymes and other reagents included in this protocol can be purchased from several well-known vendors like NEB or Thermo Fisher Scientific and perform equally well in our hands.
3. Stock concentrations of reagents could vary depending on vendor used. Adjust tables so that final concentration of reagents is the same.
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 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. Synthetic DNA targets can be used to validate specificity of padlock probes.
7. 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, anchors, and base library for sequencing. See publications for further details on probe design to target genes of interest. (see **Note 6, 7**)
9. This protocol does not go into detail on padlock probe design and analysis of data. See publications for further detail and image analysis.

Notes

1. We use ImmEdge Hydrophobic Barrier PAP Pen by Vector Laboratories (Cat. No: H-4000). Some other hydrophobic pens have shown to impede the in situ sequencing visualization.
2. Any container to hold slides in place flat and allow moisture retention (i.e. through wet whatman paper) will suffice.
3. Secure-Seal chambers come in different sizes, shapes and depths. Small chambers support ~50 µL chambers (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 volumes in protocol should be adjusted.
4. To achieve optimal optical resolution, cover glass thickness needs to be adjusted for the microscope setup used.
5. We use a Zeiss Axioimager.Z2 Epifluorescence microscope equipped with either a metal halide lamp or 6 LED light source and a Hamamatsu CCD camera. The following filter setup provides good wavelength separation and minimal crosstalk between different channels. 38HE (Zeiss) for imaging GFP/FITC/FAM dyes; SP102v2 (Chroma) for imaging Cy3 (minimal crass talk with 38HE filter); SP103v2 (Chroma) for imaging Cy3.5/TexasRed; SP104v2 (Chroma) for imaging Cy5; 49007 (Chroma) for imaging Cy7/Alexa 7.5 dyes.

6. Oligonucleotides for padlock probes, anchors, and base libraries were ordered through **Integrated DNA Technologies (IDT)**. Upon arrival, desalted oligonucleotides are resuspended to a 100 µM stock in TE buffer (pH 8.0, **IDTE**) and stored at -20°C. Sequences can be checked for secondary structure using any web-based secondary structure prediction tools (**OligoAnalyzer 3.1 tool** from IDT).
7. Padlock probe (PLP) design software is available such as ProbeMaker at **http://probemaker.sourceforge.net**/. Program allows importing single or batch cDNA targets in FASTA format for automated PLP design. User defines parameters for PLPs. Current in-house Python software package utilizes ClustalW and BLAST+ using mouse transcriptome sequences from NCBI RefSeq database.
8. Keep 0.1% (v/v) DEPC in PBS or ddH_2O for at least 1 hour at 37°C (or overnight at RT), followed by autoclaving to break down DEPC residue. DEPC inhibits RNases present in water, buffers or labware.
9. Any slides that enhance adhesion of tissue sections can be used. (Menzel Gläser SuperFrost® work very well in our hands). We commonly use 10 µm thick sections and sections should not be more than 20 µm.
10. It is recommended to use freshly prepared formaldehyde solutions in DEPC-PBS. Working solutions can be prepared form either higher concentration methanol-stabilized stock solution or from paraformaldehyde powder. Aliquots can be stored at −20°C. Do not freeze and thaw.
11. Tween-20 coats the chambers, facilitates buffer exchange and prevents formation of ”dead spaces” inside the chamber. We recommend adding buffers and solutions into a chamber when slide is slightly tilted to prevent bubble formation.
12. RNaseH has the highest activity at 37°C. It degrades RNA from mRNA/cDNA heteroduplex during the first 37°C incubation. After 30 min, sample is transferred to 45°C which is the optimal temperature for the Amp ligase. Addition of formamide into the mix lowers dsDNA stability (T_m of PLP arms/cDNAduplex). This enables extension of PLP arms that strengthens probe “locking” on cDNA and gives a good balance between arms melting and specific binding.
13. The optimal temperature for phi29 polymerase is 37°C. If RCA is performed for several hours (or over-night) at 37°C, RCPs may start to fragment what could interfere with accurate signal counting. If large RCPs are desired (thick tissue sections or those with high autofluorescence), we advise doing RCA at RT over-night. Such approach will generate very large but compact RCPs.
14. A double edge razor or forceps can be used to facilitate complete removal of the Secure-Seal chamber.
15. We typically apply <10 µL of mounting medium for single, 50 µL Secure-Seal chamber. Remove excess of the medium by gently pressing the slide against a coverslip (excess of medium will be absorbed by the paper towel). Far-red dyes are more susceptible to photobleaching. SlowFade® Gold Antifade Mountant works best in our experience.
16. CellProfiler is a great, user-friendly tool to aid biologists in image processing and analysis. With respect to presented protocol, CellProfiler offers scripts for cell segmentation (definition of the nucleus and the cytoplasm), RCP segmentation, and assignment of RCPs to individual cells or fluorescence measurements. All scripts can be implemented in automated pipeline, allowing for batch image processing. An example script for cell and RCP identification is available at CellProfiler website **http://www.cellprofiler.org**. Briefly, gray scale TIFF images (offering highest resolution, JPEG images are processed faster and can also be used) from individual fluorescence channels are loaded into the pipeline. Cells are segmented to nuclei and cytoplasm and RCPs are identified and related to neighboring cells. Finally, number of RCPs for each cell is exported as a .csv file, which can be used for post-analysis processing.

Materials

MATERIALS

- BSA-Molecular Biology Grade - 12 mg **New England Biolabs** **Catalog #**B9000S
- Nuclease-free Water
- Absolute Ethanol
- PBS
- Ethanol 70%
- Diethyl pyrocarbonate **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D5758
- T4 DNA Ligase **BLIRT** **Catalog #**EN11
- TRANSCRIPTME Reverse Transcriptase **BLIRT** **Catalog #**RT32
- RNase H **BLIRT** **Catalog #**RT34
- Uracil-DNA Glycosylase **Thermo Scientific** **Catalog #**EN0361
- 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

Other common solutions needed:

Tris-HCl (pH 8.3)
Tris-HCl (pH 7.5)
KCl
$MgCl_2$
NaCl
DTT
NAD
Triton X-100

EDTA
$(NH_4)_2SO_4$
20X SSC
Sodium phosphate
ATP

Safety warnings

See safety data sheets for proper chemical handling and waste disposal.

Formamide
Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution.

Hydrochloric acid (HCl)
Highly corrosive. Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution.

Formaldehyde/paraformaldehyde (PFA)
Known carcinogen. Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution.

DEPC (diethyl pyrocarbonate)
Harmful, use personal protective equipment.

Before start

This working protocol has been setup to work on fresh frozen mouse brain tissue. Other tissue and other species might require different pretreatment conditions.

Enzyme Buffer solutions:
Enzyme buffer solutions can be prepared prior to experiment and stored at -20°C.

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

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

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

T4 Ligase Buffer
- 500 mM Tris-HCl (pH 7.5)
- 100 mM $MgCl_2$
- 100 mM DTT

UNG Buffer (10x)
- 200 mM Tris-HCl pH 8.0
- 10 mM EDTA
- 100 mM NaCl

Other Buffers and solutions:

Hybridization Buffer (2x):
- 4x SSC
- 40% Formamide

- Nuclease-free water

(Note: Small stock can be made and stored at room temperature in the dark)

Phosphate buffered saline (1x PBS)

- 137 mM NaCl
- 10 mM sodium phosphate
- 2.7 mM KCl
- DEPC-ddH2O pH 7.4

DEPC-PBS-Tween (DEPC-PBS-T) (see **Note 8**)

- 1x PBS
- 0.05% Tween-20

DEPC-PBS (DEPC-PBS) (see **Note 8**)

- 1x PBS

Tissue section sample preparation

1 Fresh frozen tissue samples embedded in OCT compound and stored at -80°C. Tissue is cryosectioned at 10 µm and collected on Superfrost Slides and can be stored at -80°C until fixation and start of procedure.
(see **Note 9**)

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

00:03:00 Thawing

Fixation performed with freshly prepared 3% (w/v) paraformaldehyde in DEPC-PBS for 5 min at RT.
PFA solution is applied directly on top of the section.
(see **Note 10**)

Safety information

Safety precautions: paraformaldehyde

00:05:00 Fixation

3 Sections rinsed with DEPC-PBS two times (x2).

4 The tissue is permeabilized with 0.1 M HCl (in H_2O) at RT for 5 min.
Glass slide is dipped in a ~20 ml solution of 0.1 M HCl.

Safety information

Safety precautions: HCl

00:05:00 HCl treatment

5 After permeabilization, slides are washed in DEPC-PBS for 1 min at RT.

00:01:00 PBS Wash

Rinse slide 2x more times with DEPC-PBS

6 Slides are dehydrated in an ethanol (EtOH) series.

70% EtOH, RT, 1 min
00:01:00 70% EtOH

100% EtOH, RT, 1 min
00:01:00 100% EtOH

7 Slide sections are air dried.

When dry, SecureSeal hybridization chambers mounted over tissue.
Use a chamber size that covers your desired tissue.

8 Sections are rehydrated with DEPC-PBS-T and then once with DEPC-PBS.
(see **Note 11**)

In situ reverse transcription

9 Reagents for reverse transcription were 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.

10

	Reagents	**[Stock]**	**[Final]**	**1x (µl)**
	DEPC-H_2O			34.75
	Reverse Transcriptase Buffer	10x	1x	5
	dNTPs	25 mM	500 µM	1
	BSA	20 µg/µl	0.2 mg/ml	0.5
	Random Decamers	100 µM	5 µM	2.5
	Riboprotect RNase Inhibitor	40 U/µl	1 U/ml	1.25
	TranscriptME Reverse Transcriptase	200 U/µl	20 U/ml	5
	Total			50

Note: DEPC-H_2O is used to get total volume required. Adjustments in other reagents could change the amount of DEPC-H_2O needed.

11 DEPC-PBS removed and combined reagents added to seal chamber.

Chamber is sealed and slide placed in a humidity chamber.
Slide and chamber is incubated from 6 hours to overnight at 37°C.

37 °C Incubator

Barcode padlock probe hybridization and ligation

12 Reverse transcription reagents are removed and postfixation performed with 3% (w/v) paraformaldehyde in DEPC-PBS for 30 min at RT.

00:30:00 PFA fixation

13 Wash twice with DEPC-PBS-T.

14 Reagents for padlock hybridization are combined as in the table below.

15

	Reagent	[Stock]	[Final]	1x (µl)
	DEPC-H_2O			22
	Tth Ligase buffer	10x	1x	5
	KCl	1M	0.05 M	2.5
	Formamide	100%	20%	10
	Padlock Probe	0.5 µM	10 nM each	1
	BSA	20 µg/µl	0.2 µg/ml	0.5
	Tth Ligase	5 U/µl	0.5 U/µl	5
	RNaseH	5 U/µl	0.4 U/µl	4
	Total			50

Note: DEPC-H_2O is used to get total volume required. Adjustments in other reagents could change the amount of DEPC-H_2O needed.

Safety information

Safety precautions: formamide

16 DEPC-PBS-T removed and combined reagents added to seal chamber.

Chamber is sealed and placed in a humidity chamber.

Slide and chamber is incubated for 30 min at 37°C.
Enzymes and temperature (see **Note 12**)
37 °C
00:30:00

Then switched to incubation at 45°C for 1 hour.
45 °C
01:00:00

17 Then the slide is washed twice with DEPC-PBS-T.

Rolling circle amplification (RCA)

18 Reagents for rolling circle amplification (RCA) of padlock probes are combined as in the table below.

19

	Reagents	[Stock]	[Final]	1x (µl)
	DEPC-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: DEPC-H_2O is used to get total volume required. Adjustments in other reagents could change the amount of DEPC-H_2O needed.

20 DEPC-PBS-T removed and combined reagents added to seal chamber.

Chamber is sealed and incubated in humidity chamber for

4 hours at 37°C **OR** 30°C overnight.
(see **Note 13**)

03:00:00 37C incubation
37 °C **OR** 30 °C

21 After RCA, samples washed twice with DEPC-PBS-T.

Samples protected from light.

Anchor primer hybridization

22 Reagents for anchor primer hybridization are combined according to table below.

23

	Reagents	**[Stock]**	**[Final]**	**1x (µl)**
	DEPC-H_2O			24.5
	2x Hybridization buffer	2x	1x	25
	Anchor Primer	10 µM	0.5 µM	0.5

	Total			50

Note: DEPC-H_2O is used to get total volume required. Adjustments in other reagents could change the amount of DEPC-H_2O needed.

24 Reagents added to chamber and incubated 30 min at RT, in the dark.

00:30:00 Anchor Incubation

25 Slide is washed twice with DEPC-PBS-T.

Sequence by ligation (SBL)

26 Reagents for anchor primer hybridization are combined according to table below.

27

	Reagents	**[Stock]**	**[Final]**	**1x (1 µl)**
	DEPC-H_2O			39.25
	T4 Ligase buffer	10x	1x	5
	ATP	25 mM	1 mM	2
	Base #A	10 µM	0.1 µM	0.5
	Base #C	10 µM	0.1 µM	0.5
	Base #T	10 µM	0.1 µM	0.5
	Base #G	10 µM	0.1 µM	0.5
	DAPI	100 µg/ml	0.5 µg/ml	0.25
	BSA	20 µg/µl	0.2 µg/ µl	0.5
	T4 DNA Ligase	5 U/µl	0.1 U/µl	1
	Total			50

Note: DEPC-H_2O is used to get total volume required. Adjustments in other reagents could change the amount of DEPC-H_2O needed.

28 DEPC-PBS-T removed and combined reagents added to seal chamber.

Slide is incubated 1 hour at RT in the dark.

01:00:00 SBL

29 Slide is washed twice with DEPC-PBS-T.

30 Dip slides into 70% EtOH solution and incubate for 1 min.

00:01:00 70% EtOH

31 Remove the SecureSeal chamber and dip slides in 100% for 1 min
(see **Note 14**)

00:01:00 100% EtOH

Air dry the slides.

32 Apply small amount (~10 µL) of mounting medium (Slowfade or similar) and apply a cover slip.
(see **Note 15**)

Slide can be stored in the dark and at +4°C for longer term storage before imaging.

Imaging

33 Use a standard epifluorescent microscope with 20x objective.

Use DAPI staining to focus on section as a reference point to visualize RCA products.

For details of microscope setup, check referenced publications.

34 Acquire images as needed using reference points that can be used for sequential imaging of other bases later on.

RCPs are located in different focal planes, therefore, a series of images should be captured at different focal depths. The stacks of images are then merged to a single image using the maximum-intensity projection.

Stripping of anchor and base

35 After every round of base imaging, fluorophores and probes need to be stripped of the rolling circle products in order to hybridize the next base.

Remove coverslip by dipping slide into 70% EtOH until it slips off.

Note: After second and all subsequent rounds of imaging, cover slip can be removed by dipping slide into PBS, dehydration step is not needed if hydrophobic pen is used.

36 Dip slide into 100% EtOH for 1 min and then air dry the samples.

00:01:00 100% EtOH

37 Use a hydrophobic pen to draw a barrier around section.

Alternatively, mount another SecureSeal chamber.

38 Rehydrate with DEPC-PBS-T.

39 Combine reagents in the table below for the stripping of anchor and sequence primer.

40

	Reagent	[Stock]	[Final]	1x (µl)
	DEPC-H_2O			43.5
	UNG buffer	10x	1x	5
	BSA	20 µg/µl	0.2 µg/µl	0.5

	UNG	1 U/µl	0.02 U/µl	1
	Total			50

Note: DEPC-H_2O is used to get total volume required. Adjustments in other reagents could change the amount of DEPC-H_2O needed.

41 Reagents added to section and incubated at RT for 30 min.

00:30:00

42 Sections washed once with DEPC-PBS-T

43 Sections washed with 100% formamide at RT for 3 min.

00:03:00 1st Wash

Repeat an additional two times for a total of 3 times.

00:03:00 2nd Wash
00:03:00 3rd Wash

44 Sections washed twice with DEPC-PBS-T at RT

Repeat hybridization of anchor and next base.

45 Repeat **Steps 22-44** for additional required bases.

Note: If using hydrophobic pen, chamber will not be used and solutions can just be applied on top of section during incubations.

Image analysis

46 For most up to date methodolgy on image analysis, we refer to a publication from our group currently at bioRxiv:

A spatial atlas of inhibitory cell types in mouse hippocampus
Qian X, Harris KD, Hauling T, Nicoloutsopoulos D, Manchado AM, Skene N, Hjerling-Leffler J, Nilsson M.
bioRxiv 431957.
doi: https://doi.org/10.1101/431957

We also refer to the publication this protocol is based on for image analysis:

In situ sequencing for RNA analysis in preserved tissue and cells.
Ke R, Mignardi M, Pacureanu A, Svedlund J, Botling J, Wählby C, Nilsson M.
Nat Methods. 2013 Sep;10(9):857-60.
doi: 10.1038/nmeth.2563

(Cell profiler: see **Note16**)