Home made direct RNA detection

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We use this protocol and it's working

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ABSTRACT

Highly multiplexed spatial profiling of mRNAs has recently gained traction as a tool to explore cellular diversity, allowing to visualize the cell composition of tissues directly in-situ.
We propose a sensitive, open-source, flexible and customisable method for the generation of in-situ expression maps of hundreds of genes. The method exploits direct ligation of padlock probes on their target mRNAs, coupled with rolling circle amplification and hybridization-based in situ combinatorial barcoding, to achieve high detection efficiency, high throughput and large multiplexing (up to few hundred genes).

GUIDELINES

This protocol has been thoroughly tested on a variety of fresh frozen tissue sections coming from various animal models with minimal modification. Different tissues have sometimes slightly different cell or extra-cellular matrix composition and might need specific pre-treatments to make the tissue layers accessible to reagents and enzymes.

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

Necessary enzymes:

T4 RNA Ligase 2
Phi29 polymerase
RNAse Inhibitor

General Guideline and Controls

1. 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.
2. Enzymes can be produced on the cheap by your local protein production facility, if you have one nearby. The patent rights for these enzymes have expired, we provide here links to the primary sequences for your convenience.
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.

Abbreviations

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

DO: detection oligonucleotide

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 INSTRUCTIONS

Embed your fresh-frozen sample in a cryo embedding mold of your choice using OCT medium.
Cut sections to a thickness of 10-20 micra and attach the sections to Superfrost slides.
Store the sections at -80 until use.

Tissue preparation and fixation

1 Embed fresh-frozen tissues in OCT (optimal cutting temperature) compound and store them at -80°C until cryosectioning.

Section the tissues at 5-20 µm thickness and collect on SuperFrost Plus adhesion slides and store at -80°C until used for experiment.

When working with *Drosophila* tissues or other very fragile material, the tissues can be fixed for 20' in 4% PFA at room temperature, washed 4 times in PBS, cryoprotected in 30% sucrose in PBS overnight, and finally embedded in OCT. This slightly reduces the detection efficiency but keeps a much better tissue morphology.

2 Take the slides from -80°C and leave them at room temperature (RT) for 3-5 min to air dry and reach room temperature. 00:00:00

3 Fix with freshly prepared 3% formaldehyde in PBS for 5 min at RT.
Formaldehyde solution can 00:00:00 be applied directly on top of the section, or the slide can be submerged in a large amount of fixative.

Safety information

Formaldehyde! Follow the appropriate safety guidelines

4 Discard formaldehyde from tissue section and wash with PBS at RT 3 times

5 Permeabilize the tissue with 0.1 M HCl (in H2O) at RT for 5 min.
This can either be done by submerging the slide in HCl or by covering the section as in step 3

00:00:00

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. In many of the cases this doesn't seem a crucial step.

6 After permeabilisation, wash the sections with PBS two times.

7 Dehydrate the sections in order to aid 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.

8 Apply SecureSeal hybridization chambers of the appropriate size over the tissue.

9 Wash the section and the chamber by applying PBS-T to chamber inlet and then wash once with PBS. Let PBS remain in chamber until the reagents for the next steps are prepared.

The PBS-T will coat with detergent the surface of the tissue, slide and chamber, making the next steps of washing and pipetting much easier.

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.

Hybridisation of padlock probes

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Prepare the following hybridisation mix.

	A	B	C	D
	Reagent	Stock [ ]	Final [ ]	Amount

	A	B	C	D
	DEPC H2O			39.5
	20x SSC	20x	2x	5
	Formamide	100%	10%	5
	Chimeric PLPs	1 uM each	10 nm each	0.5
	Total			50

Remove the PBS from the chamber, add the hybridisation mix and incubate at 37 degrees C overnight

Safety information

Formamide

11 Remove the hybridisation mix.
Wash twice in 10% formamide in 2xSSC.
Wash twice in 2xSSC.

Probe ligation

12 Prepare the following ligation mix.

	A	B	C	D
		stock [ ]	final [ ]	
	DEPC h2O			27,25
	Hm T4 Rnl2 buffer (No Mg)	10x	1x	5
	MgCl2	25mM	2mM	4
	ATP 1 mM	1mM	100 uM	5
	RCA primer	1µM	0.05µM	2,5
	RiboProtect (RNase inhibitor)	40U/µl	1U/µl	1,25
	T4 Rnl2 (homemade)	10U/µl	0,5U/µl	2,5
	total			50

Remove the 2xSSC and add the ligation mix to the chamber.
Incubate for 2 hours at 37 degrees C.

Amplification of the padlock probes

13 Wash twice with PBS.

Prepare the following amplification mix.

	A	B	C	D
	Reagents			
	DEPC H20			34
	phi29 buffer	10X	1X	5
	glycerol (Placed at RT)	50%	5%	5
	dNTPs	25 mM	0.25mM	0,5
	BSA	20 ug/ul	0.2µg/ml	0,5
	phi29 polymerase	10 U/ul	1U/µl	5
	total			50

Incubate at 30 degree celsius O/N

Labeling

14 After overnight incubation, remove reagents from chamber.
Wash chamber twice with PBS. Let PBS remain until next step.