Imaging based barcode readout in cultured cells

This protocol is based on Askary, et al. 2020. *Nature Biotechnology* 38 (1): 66–75 for imaging barcodes in adherent cells. The readout procedure is based on seqFISH+ (Eng, et al. 2019. Nature 568 (7751): 235–39). So for more information check out the paper and this related protocol:

https://protocolexchange.researchsquare.com/article/nprot-7333/v1

We use two types of probes. Readout probes are 15nt ssDNA oligos that are coupled with a fluorescent dye. Primary probes are longer ssDNA oligos (usually around 150nt) that bind to transcribed barcode RNAs and also include sequences complementary to readout probes. Primary probes are not fluorescent themselves. This scheme provides flexibility in assigning fluorescence channels to barcodes and is cost effective. In most cases the region of primary probes that anneals to transcribed barcodes (i.e. footprint) is 20nt long. Short footprint of the primary probes makes it necessary to circularize them to reduce the chance of losing them during multiple wash and rehybridization rounds. If primary probes with longer footprint (e.g. 35nt) are used for an experiment circularization and post-fix steps in Day 2 and 3 can be skipped.

For optimal results, it is important to keep RNases away from your sample and reagents to the extent possible. Use RNase free water and buffers, spray the surfaces and your gloves with RNase Away, do not touch anything without gloves, and use filtered RNase free tips. The most common source of RNase contamination in the labs is from miniprep buffers that contain high concentrations of RNase (e.g. Buffer P1).

Cells can be cultured in a glass bottom plate or on coated coverslips. You can find the protocol for preparation of such coverslips here:

https://www.protocols.io/view/coverslips-preparation-for-seqfish-36wgq5rpogk5/v1

Day 1

Remove the media from the cells and wash once with PBS.

Fix and permeabilize the cells with MAA (3ml Methanol + 1ml Acetic Acid) at room temperature (RT) for 20min.

> MAA is 3:1 mix of methanol and acetic acid made fresh at RT.

Take MAA out and wash three times with PBS.

Start preparing the transcription mix at this point by mixing nuclease free water (NFW), NTPs, and 10X buffer at RT. However, keep the enzyme at -20°C and don't add it until ready. We usually use MEGAscript T3 Transcription Kit (ThermoFisher; Catalog number: AM1338).

> To make a transcription mix of 10µl with MEGAscript T3 enzyme: Mix 1µl of each NTP with 1µl of 10X transcription buffer, and 4µl of NFW. 1µl of enzyme is added when you are ready

to start the reaction. It is very important to use the cleanest water possible here, otherwise you not only don't get a barcode signal but also destroy the endogenous mRNAs.

Replace PBS with NFW.

Remove NFW, add the transcription mix, and incubate in a humidified chamber at 37°C for 2 hours.

Fix with 4% PFA for 15min at RT.

Wash with 2X SCC, twice for about 5min each.

Incubate with hybridization buffer for 15min at 37°C. Meanwhile prepare the hybridization buffer with probes.

> The choice of hybridization buffer depends on the application. If the goal is to detect single base pair variations, we use 30% hyb buffer from Molecular Instruments. If we only want to check the presence or absence of a barcode sequence, we usually use a 10% hyb buffer which is: 10% formamide, 10% dextran sulfate (Sigma D8906), 2X SSC in NFW.

Hybridize the primary probes at 4nM/probe final concentration in the hybridization buffer overnight at 37°C in a humid container.

Day 2

Wash 4 times at 37°C over a one hour period with a wash buffer containing the same percentage formamide as the hybridization buffer.

> Wash buffer ingredients are 10 or 30% formamide, 0.1% Triton X-100, 4X SSC in NFW. Stringency of wash should be comparable to that of hybridization. So use the same percentage of formamide as you used for hybridization, depending on the application.

Wash once with 4x SSC at RT for 5 minutes.

> You can pause at this point and leave the samples in 4x SSC with 0.02U/µl SUPERase-In RNase inhibitor (ThermoFisher AM2694) at 4°C.

If primary probes with 20nt footprint are used and sequential rounds of hybridization/imaging are needed, proceed to circularization and postfix as follows. Otherwise skip the bullet points below and continue with hybridization of readout probes.

- Hybridize samples with 50nM ligation bridge in 20% hyb buffer 4xSSC at 37°C for at least 4 hours.
    - Ligation bridge is a ssDNA oligo that bridges the two arms of the primary probes. It has a 15nt complementary sequence on each arm.

    - The hyb buffer here is: 4X SSC, 20% Formamide, 10% Dextran Sulfate (Sigma D8906) in NFW.
- Wash with 10% wash buffer (10% formamide, 0.1% Triton X-100, 4X SSC in NFW) once briefly and then for 5 minutes at room temperature.
- Wash 3 times with 1x PBS at room temperature.
- Wash with 1:1 mix of NFW and NEB Quick Ligase Reaction Buffer (2X) for 5 min at room temperature.
- Replace wash with ligation mix (25µl Quick ligase buffer, 17.5 ul NFW, 5µl 10mM ATP, 2.5µl Quick ligase) and incubate at RT overnight.

Day 3

- Wash with 10% wash buffer (10% formamide, 0.1% Triton X-100, 4X SSC in NFW) for 5 minutes at RT.
- Wash once with 4x SSC.
- Wash 3 times with 1x PBS.
- Wash once with 1x Label-IT buffer A (Mirus MIR3900; stock 10x, diluted with NFW).
- Incubate with 1x Label-IT (stock 10x) in 1x Label-IT buffer A at RT for 45 minutes
    - 37°C for 30 min works too
- Wash 3 times with 1xPBS.
- Cross-linking with 1.5 mM BS(PEG)5 in 1xPBS at room temperature for 1 hr.
    - Prepare a 15mM solution by dissolving a 1mg vial of BS(PEG)5 (ThermoFisher Cat# A35396) in 125µL of dry DMSO. Mix by repeated pipetting or by replacing the cap and vortexing. The maximum usable volume of the vial is 800µL. BS(PEG)5 is a translucent solid and will most likely not be visible at the bottom of the vial.
- Wash 3 times with 100mM Tris-HCl pH7.4, once briefly and then 5min twice.
- Wash 3 times with 4x SSC.
- Keep samples in 4x SSC with 0.02U/µl SUPERase-In RNase inhibitor (ThermoFisher AM2694) until ready for imaging.

<u>Continue here if circularization was not needed:</u>

At this point the samples are ready for cycles of readout probe hybridization and imaging. The fluorescent readout probes are hybridized at 50nM each in 10% EC buffer for 20 minutes at room temperature. See below for instructions on how to make the EC buffer.

Wash for readout probes is done in 10% Wash Buffer (10% formamide, 0.1% Triton-X 100, 4× SSC in NFW) for 5 minute at room temperature followed by 1 minute wash in 4× SSC.

Stain with DAPI diluted in 4xSSC to 1µg/ml for 10 min.

Wash with 4x SSC for three times, ~5 min each.

Image in anti-bleaching buffer.

> Anti-bleaching buffer is made up of 1% (w/v) glucose, 1:100 diluted catalase (Sigma C3155), 1.0 mg/ml Glucose oxidase (Sigma G2133), and 50mM pH8 Tris HCl in 4x SSC.
>
> First, make a 1.43x mix of Ab base:
>
> > 1M Tris HCl pH8 - 3.575mL
> >
> > 20x SSC - 14.3mL
> >
> > 0.715g of glucose
> >
> > 0.0375g trolox
> >
> > Water to 50mL
>
> Make sure to leave the Ab base overnight at 4°C after everything (except enzymes) is mixed and dissolved, to let Trolox convert into Trolox quinone which helps for anti-bleaching more. Add Catalase and Glucose oxidase before use.
>
> If imaging many samples, it is possible to make a batch of anti-bleaching buffer. However, make sure to add the anti-bleaching buffer to the sample right before imaging and replace it with 4x SSC when the imaging is done. Also, use mineral oil to cover the top of the buffer to limit the oxidation reaction - solution should be stable for days.

After each round of image acquisition the samples are washed 3 times using 55% wash buffer at room temperature for 5 minutes to strip off readout probes.

> Washing multiple times is crucial for completely stripping off readout probes. This is then followed by 2x SSC wash for 1 minutes each round.

Then, the fluorophore-coupled readout probes are incubated at 50nM each concentration at room temperature for 20 minutes in 10% EC buffer followed by 5 minute wash in 10% wash buffer and then 1 minute wash in 4X SSC and DAPI staining.

This procedure can be repeated with the next round of readout hybridization until the completion of all rounds.

Reagents

Primary probes

We usually order oligoPools from IDT as 50pmol per probe. Dissolve probes in one tube in 50µl of IDTE pH8.0 to get 1µM stock (250x).

10% wash buffer stock (1x) / *55% wash buffer stock*

	Source	Stock concentration	Final concentration	Example
UltraPure SSC	Gibco LifeTech Cat# 15557-036	20x	2x	1 ml
Formamide	Ambion Cat# AM9342	-	10% / *55%*	1 ml / *5.5ml*
TritonX-100	Sigma-Aldrich Cat# 93443	100x = 10%	0.1%	100 ul
UltraPure water				7.9 ml / *3.4ml*
			TOTAL	10 ml

100% EC

EC chunks from the original stock (Sigma-Aldrich E26258)

Put in 50ml tubes and warm up in the 42°C water bath for 30-60 min until it melts.

50% (v/v) EC solution

100% EC solution	6 mL
Ultrapure water	6 mL

Note: should NOT vortex. EC is sensitive to mechanical stress.

10% (v/v) EC solution (1.67x)

	Source	Stock concentration	Final concentration	Example	
UltraPure SSC	Gibco LifeTech Cat# 15557-036	20x	3.34x	6 mL	1.66 mL
50% EC solution	above	50% = 5x	16.7% = 1.67x	12 mL	3.33 mL
Dextran sulfate LW	Sigma-Aldrich Cat# D4911	-	16.7% (w/v)	6 g	1.66 g
UltraPure water		-	-	~18 mL	~5 mL
			TOTAL	36 mL	10 mL