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Protocol for in situ sequencing (ISS) in mouse skeletal muscle

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Protocol status: Working
We use this protocol and it's working

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Disclaimer

This protocol has been adapted from Lee et al 2023: https://doi.org/10.1101/2023.10.10.561689

Abstract

Protocol used to perform in situ sequencing (ISS) on mouse skeletal muscle.

Materials

	Item	Supplier	Catalog number
	OCT (Optimal Cutting Temperature) Compound	CellPath	KMA-0100-00A
	SuperFrost Plus Adhesion Microscope Slides Blue tab	Epredia	J1810AMNZ
	PFA	Electron Microscopy Sciences	15714
	10X PBS Buffer pH 7.4 1000mL	Invitrogen, Thermo Fisher Scientific	AM9625
	Nuclease-free water: UltraPure ‰ Distilled Water DNase/RNase Free 500mL	Invitrogen, Thermo Fisher Scientific	10977-035
	Absolute EtOH: ACS,ISO,Reag. Ph Eur Ethanol absolute for analysis	EMSURE	1.00983.1000
	SSC (20X), RNase-free	Invitrogen, Thermo Fisher Scientific	AM9763
	Lithium Dodecyl Sulfate	Sigma Aldrich	L9781-25G
	TWEEN 20	Sigma Aldrich	P1379-250ML
	2.5LT Hydrochloric acid solution 1M (1N), NIST Standard solution ready to use, Eur.Ph. , USP, BP	ThermoFisher Scientific	10487830
	Vector® TrueVIEW™ Autofluorescence Quenching Kit	Adipogen LifeSciences	VC-SP-8400-KI01
	SECURESEAL(TM) HYBRIDIZATION CHAMBERS, 0.8mm	SigmaAldrich	GBL621502-50EA
	phi29 DNA Polymerase (10 U/µL) 1000U	LifeTechnologies	EP0092
	GeneAmp™ dNTP Blend (100 mM)	LifeTechnologies	N8080261
	T4 RNA Ligase 2 (dsRNA Ligase)	NewEngland Biolabs	M0239L
	ProLong Diamond Antifade Mountant 10mL	LifeTechnologies	P36970
	Murine RNase Inhibitor, 15'000	NewEngland Biolabs	M0314L
	Rabbit anti-Laminin Antibody (0.5 mg/mL, affinity isolated antibody, buffered aqueous solution)	Merck	L9393-100UL
	Alexa Fluor® 488 AffiniPure™ Donkey Anti-Rabbit IgG (H+L)	Jackson Immuno	AB_2313584

Probe Design

1 Padlock probes can be designed in a Jupyter Notebook using the following directions:
https://github.com/Moldia/Lee_2023/tree/main/PLP_directRNA_design

Padlock probes can also be designed using dicey from the command line:
https://github.com/gear-genomics/dicey
or their website user interface (not so suitable for large scale input)
https://www.gear-genomics.com/padlock/

Additionally, complementary oligo sequences to design FISH probes or padlock probes can be taken from **https://paintshop.io/** - the padlock probes have to be assembled manually in that case.

2 **Ordering Padlock Probes**

- Publications mention the probes had been ordered as Custom RNA oligos, we could only order as Custom DNA Ultramer from Switzerland (this might be Switzerland specific?)
- Go to Custom DNA oligos
- Ultramer DNA Oligo 4nmol scale is enough
- Add /5Phos/ to 5`-
- Add r in front of last base end 3` (we need an RNA base there)
- If it's a T we will make a U out of it (there are no Ts in RNA...)
- Standard Desalting (there is no other choice)
- when ordering many targets I asked to have each padlock probe phosphorylated, and subsequently all padlock probes corresponding to one target pooled and resuspended in IDTE at 200µM

Preferably rA and rC for better fidelity of Ligase - if you have a choice of sequences select ones ending in rA or rC
https://doi.org/10.1261/rna.066753.118

Sectioning

3 Clean the cryostat with 70% EtOH. Clean the block with RNA-Zap, 70%EtOH and dry afterwards

4 Put the number of blades needed (as many as different tissues are being sectioned) on top of the block and turn on UV-lamp. Keep brushes and other tools that will be used also in the cryostat.

Careful! Do NOT put tissue in the cryostat at this time!

5 After UV-cleaning, add tissue to the cryostat, and let the tissue equilibrate to the cryostat temperature.

6 Tissue is cryosectioned at 10µm thickness and collected on SuperFrist Plus adhesion slides and can be stored at -80°C until further use.

7 Take slides with sections from -80°C and leave at room temperature (RT) for 5min to air dry (helps with tissue adherence).

8 (Fixation) Add 500µL 4% PFA in 1XPBS at RT for 30 min (under the hood). From here on slides can be fully submerged in solutions (10X Genomics slide containers are very convenient).

Representative image of plastic boxes we received for Visium experiments from 10X Genomics.
https://www.auxilab.es/en/laboratory-equipment/plastic-box-for-5-slides/

9 Discard the solution and wash 3X with 0.5% PBST (1XPBS 0.5% Tween-20)

10 (Permeabilization) Permeabilize tissue with 0.1M HCl in RNase-free H2O at RT for 5min

11 Discard the solution and wash 2x with 1XPBS.
The washing itself is important, it is less important to wash it for a particular time.

12 Submerge slides in 50% Ethanol for 5min.

13 Submerge slides in 70% Ethanol for 5min.

14 Submerge slides in 100% Ethanol for 3h at -20°C (here one can incubate over-night).

15 (Protease) Digest tissue with Protease III (RNAscope kit) for 30min at RT.

16 Wash slides 2x with 1XPBS.

17 Wipe surface around tissue section dry, apply SecureSeal hybridization chamber.

18 Add 100µL PBS-Tween (0.5% Tween-20), incubate for 1 min at RT (NOTE: PBST makes it easier for liquid to spread within hybridization chamber).

19 Remove the solution from the chamber and wash with 1XPBS. Leave the sections in 1XPBS until the next step.

Hybridisation of Padlock Probes (over-night)

20 Prepare the following mix (100µL for one slide):
Heat the probes to 92°C for 2min (maximum) and cool on ice, spin down

	Reagents	Stock concentration	Final concentration	Volume (µL)one slide	Volume (µL)
	RNase-free H2O			76.1	456.6
	SSC	20x	2x	10	60
	Formamide	100%	10%	10	60
	BSA	50µg/µL	0.2µg/µL	0.4	2.4
	RNase inhibitor	40U/µL	1U/µL	2.5	15
	Padlock probes	1µM eachPool of 1µM each	10nM each	1	6
	TOTAL			100	600

21 Remove 1XPBS from the chambers

22 Pipette the mix into the chamber and seal using the SealTabs which come with SecureSeal chambers

23 Incubate overnight at 37°C in RNAScope oven

Ligation of the padlock probes

24 Remove the hybridisation mix from the chamber and wash with 1xPBST and then 1xPBS - the chamber might start to look "dirty" on the outside–this is okay, there is no need to wipe it down all the time.

25 Prepare the following mix (100µL for one slide):

Reagents (all bought from IDT)	Stock concentration	Final concentration	Volume (µL)one slide	Volume (µL)
BSA (ultrapure)	50µg/µL	0.2µg/µL	0.4	2.4
RCA primer (adpt1 primer)(Now: SF-FWD Primer)	100µM	5µM	1	6
RNase inhibitor	40U/µL	1U/µL	1.5	15
RNase-free H2O			81.1	486.6
T4 Rn ligase 2 buffer	10x	1x	10	60
T4 Rnl2 Ligase	10U/µL	0.5U/µL	5	30
TOTAL			100	600

26 Remove 1XPBS from the chambers

27 Pipette the mix into the chamber and seal using the SealTabs which come with SecureSeal chambers

28 Incubate at 37°C in RNAscope oven for 2h

Rolling Circle Amplification

29 Turn temperature of oven down to 30°C.
Remove the ligation mix from the chamber and wash 1 x PBST and 1 x PBS

30 Prepare the following mix:

Reagents	Stock concentration	Final concentration	Volume (µL)	Volume (µL)
RNase-free H2O			68.6	411.6

Reagents	Stock concentration	Final concentration	Volume (µL)	Volume (µL)
Phi29 buffer	10x	1x	10	60
Glycerol	50%	5%	10	60
dNTPs (92C 2min)	25mM per dNTP	0.25mM	1	6
BSA	50µg/µL	0.2µg/µL	0.4	2.4
Phi29 Polymerase	10U/µL	1U/µL	10	60
TOTAL			100	600

31 Remove 1XPBS from the chambers

32 Pipette the mix into the chamber and seal using the SealTabs which come with SecureSeal chambers

33 Incubate at 30°C in RNAscope oven for 6h.

34 In the meantime thaw readout and imaging oligos and prepare Round 1 for readout + imaging oligo hybridization.

Round 1:

35 Heat Readout oligos and fluorescent adaptors at 92C for 2 min (not longer). Place back on ice. Spin down.

36 Remove the Rolling circle amplification mix from the chamber and wash 2 times with 1xPBS. Heat RNAscope oven back up to 37°C.

37 At this point, the chambers can be removed and a barrier can be drawn with a pap pen. This allows for the use of less volume.

38 After preparing the mix, filter through a 0.2um filter (you get ~half the volume).
add following mix (readout + imaging oligo hybridisation mix)

Reagents	Stock concentration	Final concentration	Volume (µL)one slide	Volume (µL)
RNase-free H2O			34	192
SSC	20x	2x	10	60
Formamide	100%	20%	20	120
Readout oligos (for adpt	10µM	0.1µM	1 µL each (33 targets = 33µL total) + 3µL	6 each(210µL total)+ 18µ

	Reagents	Stock concentration	Final concentration	Volume (µL)one slide	Volume (µL)
	1, adpt2 & adpt3) + imaging oligos: adpt1 + adpt2 + adpt3			= 36µL	L=228
	TOTAL			100	600

39 Apply readout hybridisation mix to the chamber and incubate at 37°C in RNAscope oven for 30min.

40 Discard the mix and wash 2x with 2xSSC + 0.03% LDS (Lithium Dodecyl Sulfate)

41 Add TrueVIEW and incubate for ~1min (NOT longer than 2min!!!!) - do not use detergent after this step for wash steps as it washes out TrueVIEW

42 Wash 2x with 1xPBS

43 Add DAPI (1:1000 1XPBS) for nuclei stain and incubate for 10min at RT

44 Wash 2x with 1xPBS

45 Add ~20µL of mounting media on top of the tissue - ProLong Diamond, coverslip and store in the dark at 4°C until imaging the next day.

Round 2:

46 Decoverslip in tube of 1XPBS - wait for coverslip to fall off - this could be overnight if slide had been mounted more than a day ago.
Decoverslipping in 2xSSC + 0.03% LDS also works.

47 Strip imaging oligos with 100% Formamide 2x for 2min.
Formamide is toxic and has to be discared separately. Work under the hood!

48 Wash 2x with 2xSSC + 0.03% LDS

49 Prepare the mix for R2 as per previous table (according to correct barcode combinations).

50 apply readout hybridisation mix to the chamber and incubate at 37°C in RNAscope oven for 30min - make a humidity chamber

51 Discard the mix and wash 2x with 2xSSC + 0.03%LDS

52 Add TrueVIEW and incubate for ~1min (NOT longer than 2min!!!!) - do not use detergent after this step for wash steps as it washes out TrueVIEW

53 Wash 2x with 1xPBS

54 Add DAPI (1:1000 1XPBS) for nuclei stain and incubate for 10min at RT

55 Wash 2x with 1xPBS

56 Add ~20µL of mounting media on top of the tissue - ProLong Diamond, coverslip and store in the dark at 4°C until imaging the next.

Round 3 & 4 + Membrane Stain:

57 Proceed the same for Round 3 and Round 4.

58 After hybridisation of Round 4 stain for cell-membrane markers. We stain for Laminin.

59 Remove round 4 hybridisation mix and wash 2x with 2xSSC + 0.03%LDS

60 Wash 2x with 1XPBS

61 Block section with 0.4% Triton-X, 3%BSA in 1XPBS for 30min at RT

62 Incubate in primary antibody rabbit anti-laminin (1:100 in 3% BSA in 1XPBS) for 1h at RT.

63 Wash 4x with 1XPBS

64 Incubate in secondary AB mix (Dk anti-Rb 488 1:100 in 3%BSA in 1XPB) for 1h at RT

65 Wash 4x with 1XPBS

66 Add TrueVIEW and incubate for ~1min (NOT longer than 2min!!!!) - do not use detergent after this step for wash steps as it washes out TrueVIEW

67 Wash 2x with 1xPBS

68 Add DAPI (1:1000 1XPBS) for nuclei stain and incubate for 10min at RT

69 Wash 2x with 1xPBS

70 Add ~20µL of mounting media on top of the tissue - ProLong Diamond, coverslip and store in the dark at 4°C until imaging the next.

Protocol references

Based on the original protocol in Lee et al 2023: https://doi.org/10.1101/2023.10.10.561689