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BARseq - BARseq-styled in situ sequencing for barcoded rabies virus

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We use this protocol and it's working

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Abstract

This protocol describes the application of BARseq-style in situ sequencing adapted for barcoded rabies virus. Similar procedures for both trans-synaptic tracing and retrograde tracing experiments.

Guidelines

Standard precautions with RNA samples should be taken to reduce RNA degradation during tissue processing and library preparation. Pipetting and suctioning should be gentle throughout the whole procedure, and sample should not be left dried.

Materials

DNA oligos

	A	B	C
	YS220	GATCGTCGGACTGTAGAACTCTGAACCTGTCG	sequencing primer
	YS221	/5Alex594N/GATCGTCGGACTGTAGAACTCTGAACCTGTCG	Hybridization probe to visualize all genes
	XC1417	GTTCAGAGTTCTACAGTCCGACGATC	RCA primer for SNAP25 RNA padlock
	XC2757	/5AmMC12/NNNNNNNNNNNNNNNNNNNN	N20 for random priming
	XC2758	/5Alex488N/AGTCAGCGTCGAGCACGCGGCACTTATTGCA	Hybridization probe to visualize Slc17a7
	XC2759	/5Alex532N/TGAGTAGAGTTGACTAAGAGCCGTTAGATGCC	Hybridization probe to visualize Gad1
	XC2760	/5Alex647N/TCGCTGTACTAATAGTTGTCGACAGATCGTCA	Hybridization probe to visualize B19G
	XCAI5	/5phos/agctccggcattttgttattcaTCCTCTATGATTACTGACTGCGTCTATTTAGTGGAGCCATTGCTATCTTCTTggatatacacaatccgtagattgct	pRV-4mCherry-NheI-N20 BC gapfilling padlock
	XCAI6	/5AmMC6/g+ac+at+at+tc+ga+gt+gactcataagaagt	primer 1 for pRV-4mCherry-NheI-N20 and pRV-4mCherry-CSCS2
	XCAI7	/5AmMC6/g+aa+gt+tg+aa+ta+ac+aaaatgccggagc	primer 2 for pRV-4mCherry-NheI-N20 and pRV-4mCherry-CSCS2
	XCAI63	/5AmMC6/gggagtgactgacacctccctccctg	RV B19G RT primer, Hyb XC2760 for detection
	XCAI64	/5AmMC6/tatcaacatcaaggcagtcagggccc	RV B19G RT primer, Hyb XC2760 for detection
	XCAI65	/5AmMC6/tcctgagacctgattgtgcacatcgg	RV B19G RT primer, Hyb XC2760 for detection
	XCAI66	/5AmMC6/aactccatatgttgctggaggaggga	RV B19G RT primer, Hyb XC2760 for detection
	XCAI67	/5AmMC6/tgcttttccaaacccagggacaagtt	RV B19G RT primer, Hyb XC2760 for detection
	XCAI68	/5AmMC6/tttcgtctgagcgaaagtcgtgcagg	RV B19G RT primer, Hyb XC2760 for detection
	XCAI69	/5AmMC6/ttgcatcgagacccatgttccatcca	RV B19G RT primer, Hyb XC2760 for detection

	A	B	C
	XCAI70	/5AmMC6/aggcctctttcatctacaaagccgca	RV B19G RT primer, Hyb XC2760 for detection
	XCAI71	/5AmMC6/acatccctagtctcggattctcgggc	RV B19G RT primer, Hyb XC2760 for detection
	XCAI72	/5AmMC6/aagacaccgctactcctgagcacttc	RV B19G RT primer, Hyb XC2760 for detection
	XCAI73	/5AmMC6/tggtttttacagttcgaagccagcgg	RV B19G RT primer, Hyb XC2760 for detection
	XCAI74	/5AmMC6/caccggccatcttccagttgtacgcg	RV B19G RT primer, Hyb XC2760 for detection
	XCAI75	/5AmMC6/agtgtaggtttcagcctccgtcacaa	RV B19G RT primer, Hyb XC2760 for detection
	XCAI76	/5AmMC6/atgtaggagaaccctgacaggttggt	RV B19G RT primer, Hyb XC2760 for detection
	XCAI77	/5phos/acacaatctcagagggacaggTCGCTGTACTAATAGTTGTCGACAGATCGTCACTTCGTTCCTcaatcgatcagaacctacgca	RV B19G padlock, Hyb XC2760 for detection, use with XC63
	XCAI78	/5phos/gggaagtatgtattactgagtgcTCGCTGTACTAATAGTTGTCGACAGATCGTCACTTCGTTCCTtgacttgggtctcccgaactgg	RV B19G padlock, Hyb XC2760 for detection, use with XC64
	XCAI79	/5phos/tgttgaagttcaccttcccgaTCGCTGTACTAATAGTTGTCGACAGATCGTCACTTCGTTCCTcggtgacgaggctgaggattt	RV B19G padlock, Hyb XC2760 for detection, use with XC65
	XCAI80	/5phos/tcccagagatgcaatcatcccTCGCTGTACTAATAGTTGTCGACAGATCGTCACTTCGTTCCTgacctgacggcaatgtcttaa	RV B19G padlock, Hyb XC2760 for detection, use with XC66
	XCAI81	/5phos/gtttcagacgtctcagtcattTCGCTGTACTAATAGTTGTCGACAGATCGTCACTTCGTTCCTtcatgacaaccaagtcagtga	RV B19G padlock, Hyb XC2760 for detection, use with XC67
	XCAI82	/5phos/cccgataagttggtgaacctgTCGCTGTACTAATAGTTGTCGACAGATCGTCACTTCGTTCCTaatgaaaccaaatggtgccct	RV B19G padlock, Hyb XC2760 for detection, use with XC68
	XCAI83	/5phos/tggagttctaggacttagacttTCGCTGTACTAATAGTTGTCGACAGATCGTCACTTCGTTCCTgagcatgcaaactcaagttatg	RV B19G padlock, Hyb XC2760 for detection, use with XC69
	XCAI84	/5phos/ccaaagggagtgagacttgcgTCGCTGTACTAATAGTTGTCGACAGATCGTCACTTCGTTCCTatagtagagggaagagagcat	RV B19G padlock, Hyb XC2760 for detection, use with XC70
	XCAI85	/5phos/gattacaccatttggatgcccTCGCTGTACTAATAGTTGTCGACAGATCGTCACTTCGTTCCTacctactgctccactaaccac	RV B19G padlock, Hyb XC2760 for detection, use with XC71

	A	B	C
	XCAI86	/5phos/agggtcttccctagcgggaagtTCGCTGTACTAATAGTTGTCGACAGATCGTCACTTCGTTCCTtatgacagatcccttcactcg	RV B19G padlock, Hyb XC2760 for detection, use with XC72
	XCAI87	/5phos/caatccgtaccctgactaccgTCGCTGTACTAATAGTTGTCGACAGATCGTCACTTCGTTCCTcccagatatgaagagtctctaca	RV B19G padlock, Hyb XC2760 for detection, use with XC73
	XCAI88	/5phos/ccagatgcatgtagagccgcgtTCGCTGTACTAATAGTTGTCGACAGATCGTCACTTCGTTCCTagaaagcatttccgcccaaca	RV B19G padlock, Hyb XC2760 for detection, use with XC74
	XCAI89	/5phos/gttcacttgcacaggcgttgtTCGCTGTACTAATAGTTGTCGACAGATCGTCACTTCGTTCCTtcttagccataaaagtgaacgg	RV B19G padlock, Hyb XC2760 for detection, use with XC75
	XCAI90	/5phos/tggaggacgaaggatgcaccaTCGCTGTACTAATAGTTGTCGACAGATCGTCACTTCGTTCCTgctgcccaaacaatttggtag	RV B19G padlock, Hyb XC2760 for detection, use with XC76
	XCAI131	TGGAGCCATTGCTATCTTCTTggatatacacaatccgtagattgct	sequencing primer for XCAI5

Reagents

- Phusion high-fidelity PCR kit **Thermo Scientific Catalog #**F553S
- Tween-20 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P-7949
- BS(PEG)9, 100 mg (Note: BS(PEG)9 loses its effectiveness 1 month after reconstitution in DMSO. Prepare a fresh batch every month, especially if it has been frozen and thawed repeatedly. **Thermo Scientific Catalog #**21582
- Formamide **Thermo Fisher Scientific Catalog #**AM9342
- 10x PBS **Thermo Fisher Scientific Catalog #**AM9624
- RNase-free water
- dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10mM) **Thermo Fisher Scientific Catalog #**R0192
- phi29 DNA Polymerase (10 U/µL) **Thermo Scientific Catalog #**EP0091
- RNAse H **Enzymatics Catalog #**Y9220L
- Glycerol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G5516

- Ethanol **Merck Millipore (EMD Millipore) Catalog #**100983
- Pierce™ MMTS (methyl methanethiosulfonate) **Thermo Fisher Catalog #**23011
- SSC (20X), RNase-free **Thermo Fisher Catalog #**AM9770
- RiboLock RNase Inhibitor (40 U/µL) **Thermo Fisher Catalog #**EO0381
- RevertAid H Minus Reverse Transcriptase (200 U/µL) **Thermo Fisher Catalog #**EP0452
- Paraformaldehyde 20% **Electron Microscopy Sciences Catalog #**15713
- BSA Molecular grade **New England Biolabs Catalog #**B9000S
- Ampligase DNA Ligase Kit **Lucigen Catalog #**A8101
- KCl (2 M) RNase-free **Thermo Fisher Scientific Catalog #**AM9640G
- Aminoallyl-dUTP Solution (50 mM) **Thermo Fisher Scientific Catalog #**R1101
- Tris (1 M) pH 8.0 RNase-free **Thermo Fisher Scientific Catalog #**AM9855G
- HiSeq SBS Kit v4 **Illumina, Inc. Catalog #**FC-401-4003
- Grace Bio-Labs HybriWell-FL™ sealing system Fluor-friendly adhesive chamber **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**GBL612204

Other equipment required include incubators set at 37 ″C, 45 ″C, and 60 ″C. All tubes should be RNase-free. RNase-free filter tips should be used. A Crest Xlight v3 spinning disk confocal on an Nikon Ti2E with Photometrics Kinetix, and Lumencor Celesta was used for imaging the sequencing steps. The filters and lasers used are indicated in Table 1.

	A	B	C	D
	Channels	Laser	Dichroic	Emission filter
	G/YFP	514	Zt405/514/635rpc	FF01-565/24
	T/RFP	561	FF421/491/567/659/776-Di01	FF01-441/511/593/684/817
	A	640	Zt405/514/635rpc	FF01-676/29
	C	640	Zt405/514/635rpc	FF01-775/140
	GFP	488	FF421/491/572-Di01	69401m
	DAPI	405	FF421/491/572-Di01	69401m
	TexasRed	561	FF421/491/572-Di01	69401m

	A	B	C	D
	Cy5	640	Zt405/514/635rpc	ZET532/640m

Table 1. Laser and filter settings for sequencing imaging.

Safety warnings

Use caution when handling liquids containing formaldehyde and formamide.

Library preparation

1 Tissues with barcoded neurons should be cryo-sectioned to 20 µm and mounted on slides. Slides can be stored at -80 °C for up to a month.

2 **DAY 1**

Take slide(s) out of -80 °C and immerse immediately in 4% paraformaldehyde in 1x PBS (2 slides per 50mL falcon tube, back-to-back)

3 Incubate for 1 hour at room temperature on slow shaker

4 Wash the slides by immersing in 1x PBS (2 slides per 50ml falcon tube, back to back)

5 Wipe excess PBS off the surface of the chamber, then stick on the Hybriwell-FL chambers. Note that the ports on the chamber should be placed as far away from the tissue slices as possible.

6 Wash twice in PBST (1x PBS + 0.5% Tween-20)

7 Wash in 70% Ethanol for 5 mins

8 Wash in 85% Ethanol for 5 mins

9 Wash in 100% Ethanol for 5 mins

10 Replace with new 100% Ethanol, drop extra 100% Ethanol on top of slides and cover with ParaFilm to avoid evaporation. Incubate for at least 1.5 hrs at 4 °C (up to 3 hours)

11 Wash in PBST for 4-6 times, until all bubbles are cleared in the chamber and PBST flows in and out of the chamber smoothly.

12 Make reverse transription mix: 50 µM N20 primer (XC2757), 2 µM XCAI6, 2 µM XCAI7, 20 U/µL RevertAid H Minus M-MuLV reverse transcriptase, 500 µM dNTP, 0.2 µg/µL BSA, 1 U/µL RiboLock RNase Inhibitor, 1x RevertAid RT buffer.

For the monosynaptic tracing experiments, the RT primers additionally included 2 µM of primers for the rabies glycoprotein (XCAI63 through XCAI76).

13 Incubate in reverse transcription mix overnight at 37 °C. Create a humidity chamber to avoid the slides drying out using kim-wipes and DI water.

14 **DAY 2:**

Wash with PBST once

15 Incubate in a mixture of 1µL BS(PEG)9 per 4 µL PBST (e.g. 200ul BS(PEG)9 and 800ul PBST) for one hour at room temperature

16 Wash with 1M Tris pH 8.0, then incubate in new 1M Tris pH 8.0 for 30 mins

17 Wash twice in PBST

18 Make non-gap-filling ligation mix: 1x Ampligase buffer, 20 nM padlock probe each, 0.5 U/µL Ampligase, 0.4 U/µL RNase H, 1 U/µL RiboLock RNase Inhibitor, 50 mM KCl (extra of those already provided by the ampligase buffer), 20% formamide.

In the retrograde tracing experiments, the non-gap-filling padlock probe mix included all padlock probes for endogenous genes. In the monosynaptic tracing experiments, the non-gap-filling padlock probe mix included all padlock probes for endogenous genes except *Slc30a3*, and additionally included padlocks for the rabies glycoprotein (XCAI77 – XCAI90).

19 Incubate in ligation mix for at least 30 mins at 37 °C (can go longer but not shorter), then at least 45 mins at 45 °C (can go longer but not shorter).

20 Make the gap-filling ligation mix [same as the non-gap-filling mix with the rabies barcode padlock probe (XCAI5) as the only padlock probe, and with 50 µM dNTP, 0.2 U/µL Phusion DNA polymerase, and 5% glycerol]

1x Ampligase buffer, 20 nM padlock probe each, 0.5 U/µL Ampligase, 0.4 U/µL RNase H, 1 U/µL RiboLock RNase Inhibitor, 50 mM KCl (extra of those already provided by the ampligase buffer), 20% formamide, 50 µM dNTP, 0.2 U/µL Phusion DNA polymerase, and 5% glycerol.

21 Wash twice in PBST

22 Incubate in ligation mix for 5 mins at 37 °C, then 45 mins at 45 °C. **exact timing on this step!**

23 Wash twice in PBST, then once in FISH Wash (2x SSC with 10% formamide)

24 Hybridize with 1 µM RCA primer (XC1417) in FISH wash for 10 mins at room temperature

25 Wash twice in FISH wash, then twice in PBST

26 Make RCA mix: 1 U/µL phi29 DNA polymerase, 1x phi29 polymerase buffer, 0.25 mM dNTP, 0.2 µg/µL BSA, 5% glycerol (extra of those from the enzymes), 125 µM aminoallyl dUTP

27 Incubate in RCA mix overnight at room temperature

28 **DAY 3:**

Wash with PBST once

29 Incubate in a mixture of 1µL BS(PEG)9 per 4 µL PBST (e.g. 200ul BS(PEG)9 and 800ul PBST) for one hour at room temperature

30 Wash with 1M Tris pH 8.0, then incubate in new 1M Tris pH 8.0 for 30 mins

31 Wash twice in PBST

Sequencing

32 **Hybridization of Gene sequencing primer:**

Wash with FISH wash (2x SSC with 10% formamide)

33 Hybridize sequencing primer (YS220) with a primer concentration of 1 µM in FISH wash for 10 mins at room temperature

34 Wash with FISH wash three times, 2 mins each

35 Wash with PBST twice

36 **Sequence first cycle (genes and barcodes):**
Do the following incubations. Unless noted with incubation temperature, each step is performed at room temperature. For steps without incubation time, treat these as quick washes. large flat metal blocks can be used to place sample slides to quickly cycle through high and low temperatures. This version uses MiSeq Nano v2 kit: https://www.illumina.com/products/by-type/sequencing-kits/cluster-gen-sequencing-reagents/miseq-reagent-kit-v2.html

36.1 Incorporation Buffer 60 °C 3 mins x1

36.2 2% PBST x1

36.3 Idoacetamide blocker: For 9.3mg vial dilute pellet in between 2.5-3.5mL 2% PBST. Make fresh tube daily and store out of light.

Idoacetamide blocker 60 °C 3 mins x1

36.4 2% PBST x1

36.5 Incorporation Buffer x2

36.6 IRM 60 °C 3 mins x2

36.7 2% PBST x1

36.8 2% PBST 60 °C 3 mins x4

36.9 Replace PBST with USM and Image **if slides are dirty, clean with 70% Ethanol before adding USM**

37 **Sequence subsequent cycles (genes and barcodes):**
Do the following incubations. Unless noted with incubation temperature, each step is performed at room temperature. For steps without incubation time, treat these as quick washes. large flat metal blocks can be used to place sample slides to quickly cycle through high and low temperatures.

37.1 Incorporation buffer x2

37.2 CRM 60 °C 3 mins x2

37.3 Incorporation buffer x1 - Wipe ports after adding the incorporation buffer, to ensure that no CRM is left on the slide's surface

37.4 2% PBST x1

37.5 Idoacetamide blocker: For 9.3mg vial dilute pellet in between 2.5-3.5mL 2% PBST. Make fresh tube daily and store out of light.

Idoacetamide blocker 60 °C 3 mins x1

37.6 2% PBST x1

37.7 Incorporation buffer x2

37.8 IRM 60 °C 3 mins x2

37.9 2% PBST x1

37.10 2% PBST 60 °C 3 mins x4

37.11 Replace 2% PBST with USM and image **if slides are dirty, clean with 70% Ethanol before adding USM**

38 **After completing all gene sequencing imaging cycles:**

Hybridization cycle

38.1 **Hybridize probes:**
Make strip buffer: 60% formamide 2xSSC 0.01% Tween20

Strip buffer 60 ˚C 5 mins x3
Cool down quickly on metal plates between washes, place on metal plates in 60 ˚C oven to heat up quickly.

38.2 FISH wash (2x SSC with 10% formamide) 1x

38.3 Hybridize probes (YS221, XC2758, XC2759, XC2760) with a primer concentration of 1 µM in FISH wash at 60 ˚C for 2 minutes, then for 10 mins at room temperature. Rotate plates in holder to ensure they cool down slowly.

38.4 FISH wash x1

38.5 0.002 mg/ML DAPI in PBST, room temperature for 5 mins

38.6 Replace PBST with USM and image **if slides are dirty, clean with 70% Ethanol before adding USM**

39 **After completing all gene sequencing imaging cycles and hybridization cycle:**

Hybridize barcode sequencing primers

39.1 Strip buffer (60% formamide 2xSSC 0.01% Tween20)
60 ˚C 5 mins x3
Cool down quickly on metal plates between washes, place on metal plates in 60 ˚C oven to heat up quickly.

39.2 FISH wash (2x SSC with 10% formamide) 1x

39.3 Hybridize probe XCAI131 with a primer concentration of 1 µM in 2x SSC and FISH wash at 60 ˚C for 2 minutes, then for 10 mins at room temperature. Rotate plates in holder to ensure they cool down slowly.

39.4 FISH wash x1

40 Sequencing barcodes: The sequencing procedures are the same as those for gene sequencing (Steps 36 and 37)

After hybridizing barcode primers, go directly back to step 36, sequence first barcode imaging cycle. Then repeat step 37 for desired length of barcode sequence.