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BICCN_DART-FISH

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Chien-Ju Chen[1]

[1]University of California, San Diego

Chien-Ju Chen

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We use this protocol and it's working

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Abstract

This protocol documents DART-FISH procedures used to generate spatial transcriptomic data from human brain section for BICCN.

Materials

Reagents

	A	B	C
	material	vendor	catalog number
	UltraPure™ DEPC-treated Water 1L	ThermoFisher Scientific	750023
	Pierce™ 16% Formaldehyde (w/v), Methanol-free	ThermoFisher Scientific	28908
	PBS - Phosphate-Buffered Saline (10X) pH 7.4, RNase-free	ThermoFisher Scientific	AM9624
	Ethyl alcohol, Pure	Sigma-Aldrich	E7023
	Triton™ X-100 solution	Sigma-Aldrich	93443
	pepsin	Sigma-Aldrich	10108057001
	SuperScript™ IV Reverse Transcriptase	ThermoFisher Scientific	18090010
	Advantage® UltraPure dNTP Combination Kit	ClonTech	639132
	RNase inhibitor	Enzymatics	Y9240L
	Aminoallyl dUTP, 4 mM in TE buffer *UltraPure Grade*, Anaspec Inc	Anaspec (VWR)	AS-83203
	BS(PEG)9	ThermoFisher Scientific	21582
	UltraPure™ DNase/RNase-Free Distilled Water	TheroFischer Scientific	10977023
	Ribonuclease; RNAse H; Conc. 5,000 U/mL; 5,000 U; incl. 10X buffer	Fisher Scientific	50305945
	RNase Cocktail™ Enzyme Mix	Invitrogen	AM2288
	Ampligase® Enzyme and Buffer	VWR	76081-598
	SSC (20X), RNase-free	ThermoFisher Scientific	AM9763
	Formamide (Deionized)	ThermoFisher Scientific	AM9342
	BSA, Molecular Biology Grade	NEB	B9000S
	Phi29 DNA polymerase (10U/uL)	ThermoFisher Scientific	EP0094

	A	B	C
	Acryloyl-X, SE in DMSO	ThermoFisher Scientific	A20770
	Acrylamide/Bis-acrylamide, BioReagent, for molecular biology, 37:1 (ratio)	Sigma-Aldrich	A6050-100ML
	Ammonium persulfate	Sigma-Aldrich	A3678
	TEMED	Fisher Scientific	17919
	gel slick solution	Lonza	50640
	TrueBlack lipofuscin autofluorescence quencher	Biotium	23007
	Silicone Isolators JTR20R-2.5 20mm DIA x 2.4 mm Depth 25 × 25mm OD No PSA	Grace Bio-Labs	664304
	Coverslips, Glass, 18mm dia.	Ted Pella	260369
	Azer Scientific cover glass, No. 1.5, 24 × 60 mm	Neta Scientific	Azer-1152460

Probes

	A	B	C
	oligo name	vendor	sequence
	5N_dc10-Cy5_N9	IDT	/5AmMC12/CCGATAGTCACGATCTGTGGNNNNNNNN*N
	dT20_dc7-488	IDT	CATGGATTCGCGGAGGATCATTTTTTTTTTTTTTTTTT*T
	FISSEQ_ppRCA primer	IDT	GATATCGGGAAGCTGA*A*G
	DARTFISH_anchor_Cy3	IDT	/5Cy3/CTTCAGCTTCCCGATATCCG
	dcProbe7-AF488	IDT	/5Alex488N/TGATCCTCCGCGAATCCATG
	dcProbe10-ATTO647N	IDT	/5ATTO647NN/CCACAGATCGTGACTATCGG
	dcProbe0-AF488	IDT	/5Alex488N/TGTATCGCGCTCGATTGGCA
	dcProbe0-Cy3	IDT	/5Cy3/CGTATCGGTAGTCGCAACGC
	dcProbe0-ATTO647N	IDT	/5ATTO647NN/ACGCTACGGAGTACGCCACT
	dcProbe1-AF488	IDT	/5Alex488N/TCTTGCGTGCGATACGGAGT
	dcProbe1-Cy3	IDT	/5Cy3/AACGGTATTCGGTCGTCATC
	dcProbe1-ATTO647N	IDT	/5ATTO647NN/CTGGTTCGGGCGTACCTAAC
	dcProbe2-AF488	IDT	/5Alex488N/AGAACTTGCGCGGATACACG

	A	B	C
	dcProbe2-Cy3	IDT	/5Cy3/CTACTTCGTCGCGTCAGACC
	dcProbe2-ATTO647N	IDT	GACGAACGGTCGAGATTTAC/3ATTO647NN/
	dcProbe3-AF488	IDT	/5Alex488N/GAATTGTCCGCGCTCTACGA
	dcProbe3-Cy3_2	IDT	/5Cy3/TCGTACTTCGACGGCACTCA
	dcProbe3-ATTO647N	IDT	/5ATTO647NN/AACTGCGACCGTCGGCTTAC
	dcProbe4-AF488	IDT	/5Alex488N/CGGAATACGTCGTTGACTGC
	dcProbe4-Cy3	IDT	/5Cy3/TACCATTCGCGTGCGATTCC
	dcProbe4-ATTO647N_2	IDT	/5ATTO647NN/ACTCTACCGGCAATCGCGTC
	dcProbe5-AF488	IDT	/5Alex488N/GAGTGTCGCGCAACTTAGCG
	dcProbe5-Cy3	IDT	/5Cy3/ACGTCTGCGTACCGGCTTAG
	dcProbe5-ATTO647N	IDT	/5ATTO647NN/CATGCGATTAACCGCGACTG
	dcProbe6-AF488_2	IDT	/5Alex488N/CTTGCGGCGACAGTCGAACA
	dcProbe6-Cy3	IDT	/5Cy3/TCGTAACCCGTGCGAAGTGC
	dcProbe6-ATTO647N	IDT	/5ATTO647NN/CTCTCGTAGCGTGCGATGAG
	dcProbe7-AF488_2	IDT	/5Alex488N/TTAGGTCGCCTACCGACTGC
	dcProbe7-Cy3	IDT	/5Cy3/GCCACATCGACTCGGTCTAT
	dcProbe7-ATTO647N	IDT	GCTCAGCCGGACGAGTAGAT/3ATTO647NN/

Troubleshooting

preparation

10m

1 Make sure that the padlock probes have 5' phosphate. In case they do not, run T4 PNK reaction and clean up the product using Zymo ssDNA/RNA clean up columns.

2 Wash, dry and UV the silicone isolators. UV the EasyDip jars. Move away unused stuff from the bench and clean and undust the working area. Set the HybEZ oven 37C.

permeabilization

3 Prepare 75ml of 4% formaldehyde in 1x PBS. Requires two vials of 16% formaldehyde.

	A	B
	component	volume (mL)
	DEPC-water	52
	16% PFA	20
	10X PBS	8

4 Take the sections that are on 25mm*60mm coverslips out of -80C on dry ice, insert them into the slide holder, submerge the slide holder in the staining jar containing 4% PFA in PBS. Fix for 60mins at 4C.

1h

01:00:00 4C

5 Remove the DEPC-PSBT jar and the 4% PFA jar containing the samples from 4C. Insert the sample holder into the cold 1x PBST jar. Incubate for 3 minutes. Then insert the sample holder in the room temperature 1x PBST jar. Incubate for 3 minutes.

6m

00:03:00 in 4C DEPC-PBST with occasional agitation

00:03:00 in RT DEPC-PBST with occasional agitation

dehydration and mounting

20m

6 Prepare jars of 50%, 70% and two 100% ethanol. Dehydrate Section with

20m

00:05:00 50% EtOH at room temperature

00:05:00 70% EtOH at room temperature

00:05:00 100% EtOH at room temperature

00:05:00 100% EtOH at room temperature

7 Take the coverslips out of the sample holder. 5m

00:05:00 Air drying at room temperature.

In the meantime, put 20mm diameter Press-To-Seal silicone isolators on a kipwipe on a flat surface. Carefully put the coverslips on silicone isolators, with the tissue sample in the hole. Gently press on the back of the coverslip to seal completely. You may want to stack a 20mm diameter isolator on top of the already mounted 20mm isolator to increase the volume.

permeabilization 10m

8 Permeabilize sample with **0.25% Triton X-100** in **DEPC-1X PBS** for 10m

00:10:00 at Room temperature

875 µL DEPC-H2O | 100 µL 10x PBS | 25 µL 10% Triton-X 100

	A	B
	component	x1 volume (uL)
	DEPC-water	875
	10X PBS	100
	10% Triton X-100	25

9 Wash thrice with DEPC-1X PBS and DEPC-Water

1 mL DEPC-1X PBS quick wash

1 mL DEPC-1X PBS quick wash

1 mL DEPC-water quick wash

pepsin digestion 10m

10 Digest with 0.01% Pepsin in 0.1N HCl. Pre-warm the pepsin to 1m 30s

37 °C for at least 5 minutes before use.

100 µL 0.01% Pepsin in 0.1N HCl for 00:01:30 at 37 °C

	A	B
	component	x1 volume (uL)

	A	B
	1% pepsin	1
	0.1N HCl	99

11 Wash twice with DEPC-1X PBS

1 mL DEPC-1X PBS quick wash

1 mL DEPC-1X PBS quick wash

reverse transcription

15m

12 Prepare Reverse Transcription Mix on Ice.

15m

	A	B
	component	x1 volume (uL)
	DEPC-water	90.75
	5X SSIV buffer	30
	10mM dNTP	3.75
	100uM N5_dc10-Cy5_N9	3.75
	100uM dT20_dc7-488	3.75
	0.1M DTT	7.5
	4mM aminoallyl-dUTP	1.5
	RNase inhibitor (40U/uL)	1.5
	SuperScript IV Reverse Transcriptase	7.5
	total	150

Incubate human brain sections with the Reverse Transcription Mix

150 µL Reverse Transcription Mix for 00:10:00 at 4 °C then

Overnight at 37 °C

13 Wash with 1X PBS twice.

1 mL 1X PBS quick wash

1 mL 1X PBS quick wash

cDNA crosslinking

1h 30m

14 Add the crosslinking mix to crosslink cDNAs with BS(PEG)9. Prepare 5mM BS(PEG)9 in PBS.

1h 30m

	A	B
	component	x1 volume (uL)
	250mM BS(PEG)9	10
	10X PBS	50
	ultrapure water	440

Crosslink cDNA with BS(PEG)9

500 µL 5 mM BS(PEG)9 in PBS for 01:00:00 at Room temperature

Wash with PBS twice

1 mL 1X PBS quick wash

1 mL 1X PBS quick wash

Quench unreacted crosslinker with 1M Tris, pH 8.0

1 mL 1M Tris pH8.0 for 00:30:00 at Room temperature

Wash with PBS twice

1 mL 1X PBS quick wash

1 mL 1X PBS quick wash

RNase digestion

1h

15 Prepare RNase Digestion Mix

1h

	A	B
	component	x1 volume (uL)
	ultrapure water	168
	10X RNase H buffer	20
	RNase H (5U/uL)	10

	A	B
	RNase Cocktail	2

Add 200 µL RNase Digestion Mix

Incubate at 37 °C for 01:00:00 . Cover the silicone wells.

16 Wash samples with 1X PBS twice.

1 mL 1X PBS quick wash

1 mL 1X PBS quick wash

padlock probe hybridization

33m

17 Prepare the 484-gene-Hybridization Mix according to the table below. Preheat the probe-water mix to 85 °C for 00:03:00 and immediately move them to a cold block or on ice. Then complete the Hybridization Mix. 3m

484-gene-Hybridization Mix

	A	B
	component	x1 volume (uL)
	ultrapure water	98.2
	10X Ampligase buffer	15
	HB_Feb2022 probes (27.2 ng/uL)	25.3
	100nM PLP1 oligos	1.5
	Ampligase (5U/uL)	10

add 150 µL 484-gene-Hybridization mix to human section

18 Incubate samples in the Hybridization Mix at 37 °C for 00:30:00 , then at 30m

55 °C Overnight .

For the overnight incubation, first set the Ez hyb oven to 60 °C and then change it to

55 °C as you put the samples in. Cover the sample well.

19 Wash samples with 1x PBS twice.

1 mL 1X PBS quick wash

1 mL 1X PBS quick wash

RCA

6h

20 Prepare RCA Primer Mix

1h

	A	B
	component	x1 volume (uL)
	ultrapure water	119
	20X SSC	20
	formamide	60
	100 uM FISSEQ_ppRCA primer	1

add 200 µL RCA Primer Mix to each sample and incubate at 37 °C for 01:00:00

21 Wash samples with 2xSSC.

1 mL 2X SSC quick wash

1 mL 2X SSC quick wash

22 Prepare RCA Enzyme Mix on ice.

5h

	A	B
	component	x1 volume (uL)
	ultrapure water	119.25
	10X Phi29 polymerase buffer	15
	10mM dNTP	3.75
	4mM aminoallyl-dUTP	1.5
	NEB BSA (20mg/mL)	7.5
	ThermoFisher Phi29 polymerase	3

	A	B
	total	150

Add 150 µL RCA Enzyme Mix to each sample

Incubate samples in RCA Enzyme Mix at 30 °C for 05:00:00

23 Wash samples with 1x PBS twice.

1 mL 1X PBS quick wash

1 mL 1X PBS quick wash

rolony crosslinking

1h 30m

24 Crosslink with Acryloyl-X Mix and embed the sample in gel. 1h 42m

Prepare Acryloyl-X Mix

	A	B
	Component	x1 volume (uL)
	10X PBS	50
	10mg/mL Acryloyl-X, SE in DMSO	10
	ultrapure water	440

add 500 µL Acryloyl-X Mix to the sample and incubate for 00:30:00 at Room temperature

quick wash with 1xPBS.

1 mL 1X PBS quick wash

Incubate the sample with 300 µL Acrylamide Solution for 00:30:00 at Room temperature .

Prepare Acrylamide Solution.

	A	B
	Component	x1 volume (uL)
	10X PBS	50
	40% Acrylamide/Bis (37:1)	50

	A	B
	ultrapure water	400

Aspirate the Acrylamide Solution. Do not wash the samples!!!

Prepare Polymerization Mix.
Prepare 5% TEMED: 5 µL TEMED in 95 µL ultrapure water
Prepare 4% APS: 10 mg Ammonium Persulfate in 250 µL ultrapure water
RNaseZap and UV 18mm coverslips and treat them with Gel-Slick.

	A	B
	component	x1 volume (uL)
	Acrylamide solution	138
	4% APS	6
	5% TEMED	6

Add 30 µL Polymerization Mix to the sample and cover with Gel-Slick-treated coverslip for 00:30:00 at Room temperature in an Argon tank.

wash with 1x PBST for 3min twice
1 mL 1X PBST for 00:03:00
1 mL 1X PBST for 00:03:00

Carefully remove the Gel-Slick-treated coverslip on the samples

wash with 1xPBST for 3min twice
1 mL 1X PBST for 00:03:00
1 mL 1X PBST for 00:03:00

imaging 11m

25 stain the sample with Probe Hybridization Mix with decoding probes (Take dcProbe0_AF488, dcProbe0_Cy3, dcProbe0_ATTO647N probes as an example). 8m

Prepare Probe Hybridization Mix

	A	B
	component	x1 volume (uL)
	100 uM dcProbe0_AF488	1
	100 uM dcProbe0_Cy3	1
	100 uM dcProbe0_ATTO647	1
	100% formamide	60
	20X SSC	20
	ultrapure water	117

add 200 µL Probe Hybridization Mix to each sample. Incubate for 00:08:00 at Room temperature

26 Wash with10% formamide in 2X SSC twice. Then, image. 6m

1 mL 10% formamide in 2X SSC for 00:03:00

1 mL 10% formamide in 2X SSC for 00:03:00

27 Strip with 1 mL 80% formamide in 2X SSC for 00:05:00 . 5m

Wash with 1 mL 2X SSC buffer once.

Repeat the decoding imaging with the next decoding probes (dcProbe1, dcProbe2, dcProbe3, dcProbe4, dcProbe5, dcProbe6, and dcProbe7).

28 After images of samples stained with dcProbe0, 1, 2, 3, 4, 5, 6, 7 were taken, take the nuclei staining images with Draq5 staining. 9m

add 500 µL Draq5 solution to the sample. Incubate for 00:05:00 at Room temperature

wash the sample with 1x PBS twice. Then, image.

1 mL 1X PBS for 00:02:00

1 mL 1X PBS for 00:02:00