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DART-FISH Protocol

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Chien-Ju Chen¹, Kian Kalhor¹, Kun Zhang¹

¹University of California, San Diego



Chien-Ju Chen

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We use this protocol and it's working

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Abstract

In the manuscript Mapping Human Tissues with Highly Multiplexed RNA in situ Hybridization (<https://doi.org/10.1101/2023.08.16.553610>), we describe a highly multiplexed in situ hybridization technique based on in situ padlock probe capture and demonstrate in applicability to different human tissue types. This protocol details the colony generation and decoding steps of DART-FISH.

Materials

Reagents

	Material	Supplier	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
	SUPERase-In RNase inhibitor	ThermoFisher Scientific	AM2696
	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	ThermoFisher 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

Material	Supplier	Catalog Number
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

Oligo name	Vendor	Sequence
Acr_dc10-Cy5_N9	IDT	/5AmMC12/CCGATAGTCACGATCTGTGGNNNNNNNNN*N
Acr_dc7-488_dT20	IDT	/5Acryd/CATGGATTCGCGGAGGATCATTTTTTTTTTTTTTTTV* N
rca_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/AACGGTATTTCGGTCGTCATC
dcProbe1-ATTO647N	IDT	/5ATTO647NN/CTGGTTCGGGCGTACCTAAC
dcProbe2-AF488	IDT	/5Alex488N/AGAACTTGCGCGGATACACG



	Oligo name	Vendor	Sequence
	dcProbe2-Cy3	IDT	/5Cy3/CTACTTCGTGCGTCAGACC
	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/TACCATTGCGGTGCGATTCC
	dcProbe4-ATTO647N_2	IDT	/5ATTO647NN/ACTCTACCGGCAATCGCGTC
	dcProbe5-AF488	IDT	/5Alex488N/GAGTGTGCGCAACTTAGCG
	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/

Before start

Prepare fresh frozen tissue sections at 10um thickness on coverslips. Store the sections in -80C and transfer on dry ice upon the start of the protocol.

Make sure that the padlock probes have 5' phosphate. The enzymatic production of padlock probes (accompanying protocol) leaves a 5' phosphate. If probes are individually synthesized without 5' phosphate, run T4 PNK reaction and clean up the product using Zymo ssDNA/RNA clean up columns.



Preparation

10m

- 1 Wash, dry and UV the silicone isolators. UV the EasyDip jars. Move away unused stuff from the bench. Wipe the working area by 70% ethanol and RNase Zap. Set the HybEZ oven 37 °C .
- 2 Prepare two jars of DEPC-1xPBST and keep one at 4 °C .

Component	Volume (ml)
10x PBS	8
10% Tween-20	0.8
DEPC-H2O	72

Fixation

- 3 Prepare 80ml of 4% formaldehyde in 1x PBS. Store at 4 °C for use in the same day.

Component	Volume (ml)
DEPC-water	52
16% PFA	20
10X PBS	8

- 4 Take the tissue sections that are on 25mm*60mm coverslips out of -80 °C freezer, put them on dry ice, quickly insert them into the EasyDip slide holder, submerge the slide holder in the staining jar containing 4% PFA in PBS. Fix for 01:00:00 at 4 °C .

2h

01:00:00 4C

- 5 Remove the DEPC-PBST 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 tissue sections 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. Attach another 20mm diameter isolator on top of the already mounted 20mm isolator to increase the volume.

permeabilization

10m

- 8 Permeabilize the tissue section with **0.25% Triton X-100** in **DEPC-1X PBS**

00:10:00 at Room temperature

Component	1x volume (ul)
10X PBS	40
DEPC-H ₂ O	350
RNase inhibitor (enzymatics) (40U/ul)	2
10% Triton X-100	10
SUPERase In (20U/ul)	1

- 9 Wash thrice with cold PBSTR and cold DEPC-Water

6m

200 µL PBSTR for 00:03:00

200 µL PBSTR for 00:03:00

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

Component	1x volume (ul)
1% pepsin	1
0.1N HCl	99

- 11 Wash two times with cold PBSTR ⌚ 00:00:00 🧪 200 µL PBSTR for ⌚ 00:03:00

3m

reverse transcription

15m

- 12 Prepare Reverse Transcription Mix on Ice.

15m

Component	1x volume (ul)
DEPC-H ₂ O	88.125
Acr_dc10-Cy5_N9 (100uM)	3.75
Acr_dc7-488_dT20 (100uM)	3.75
5X SSIV Buffer	30
0.1 M DTT	7.5
10 mM dNTP	3.75
4 mM aminoallyl-dUTP	1.5
RNase Inhibitor (enzymatics, 40U/ul)	3.75
Suprase In (20U/ul)	0.375
SuperScript IV Reverse Transcriptase	7.5

Incubate tissue sections with the Reverse Transcription Mix

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

⌚ Overnight at 🌡️ 37 °C

Make sure to fully cover the silicone well. If you use a coverslip, do not press on it and do not let the coverslip come in contact with the reagents inside the well.

- 13 Wash two times with cold PBSTR



🧴 200 μ L PBSTR quick wash

🧴 200 μ L PBSTR quick wash

cDNA crosslinking and gel embedding

1h 30m

14 Treat the sample with Acryloyl-X mix:

30m

Component	1x volume (ul)
10X PBS	50
10mg/mL Acryloyl-X, SE in DMSO	10
ultrapure water	440

add 🧴 500 μ L Acryloyl-X Mix to the sample and incubate for ⌚ 00:30:00 at

🌡 Room temperature

quick wash with PBSTR

🧴 400 μ L PBSTR quick wash

15 Incubate the sample with 🧴 300 μ L Acrylamide Solution for ⌚ 00:30:00 at

30m

🌡 Room temperature .

Prepare Acrylamide Solution.

Component	1x volume (ul)
10X PBS	50
40% Acrylamide/Bis (37:1)	50
SUPERase-In RNase inhibitor(20U/uL)	1.25
Enzymatic RNase inhibitor	2.5
ultrapure water	400

In the mean time prepare 5% TEMED: 🧴 5 μ L TEMED in 🧴 95 μ L ultrapure water

Prepare 4% APS: 🧴 10 mg Ammonium Persulfate in 🧴 250 μ L ultrapure water

RNaseZap and UV 18mm coverslips and treat them with Gel-Slick.

16 Prepare the polymerization mix and mix well by gently pipetting up and down.

42m

Note: Be quick at this step



	Component	1x volume (uL)
	Acrylamide solution	138
	4% APS	6
	5% TEMED	6

Aspirate the acrylamide solution and add  30 μ L Polymerization Mix to the sample and immediately cover with Gel-Slick-treated coverslip for  00:30:00 at  Room temperature in a dark Argon chamber.

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 using a needle

Wash with 1xPBST for 3min twice

 1 mL 1X PBST for  00:03:00

 1 mL 1X PBST for  00:03:00

RNase digestion

1h

17 Prepare RNase Digestion Mix

1h

	Component	1x volume (ul)
	ultrapure water	168
	10X RNase H buffer	20
	RNase H (5U/uL)	10
	RNase Cocktail	2

Add  200 μ L RNase Digestion Mix

Incubate at  37 $^{\circ}$ C for  01:00:00 . Cover the silicone wells.

18 Wash samples with 1X PBS twice.

6m



🧪 1 mL 1X PBS for ⌚ 00:03:00

🧪 1 mL 1X PBS for ⌚ 00:03:00

padlock probe hybridization

33m

- 19 Prepare the padlock-probe-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 padlock-probe-hybridization mix.

3m

Note: Adjust the volume and ultrapure water and padlock probes so that the final concentration of padlock probes is at 100 nM for the brain probe set and 180 nM for the kidney probe set

padlock-probe-hybridization mix

Component	1x volume (ul)
ultrapure water	93.1
10X Ampligase buffer	15
padlock probes (22.9 ng/uL)	31.9
100nM PLP1 oligos	1.5
Ampligase (5U/uL)	10

add 🧪 150 µL padlock-probe-hybridization mix to tissue sections

- 20 Incubate samples in the padlock-probe-hybridization mix at 🌡️ 37 °C for ⌚ 00:30:00 , then at 🌡️ 55 °C ⌚ Overnight .

30m

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 so that the tissue sections won't dry out overnight.

- 21 Wash samples with 1x PBS twice.

🧪 1 mL 1X PBS for ⌚ 00:03:00

🧪 1 mL 1X PBS for ⌚ 00:03:00

6m

RCA

6h

- 22 Prepare RCA Primer Mix

1h



Component	1x volume (ul)
ultrapure water	119
20X SSC	20
formamide	60
100 uM rca_primer	1

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

23 Wash samples with 2xSSC.

6m

 1 mL 2X SSC for  00:03:00

 1 mL 2X SSC for  00:03:00

24 Prepare RCA Enzyme Mix on ice.

7h

Component	1x 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

Add  150 μ L RCA Enzyme Mix to each sample

Incubate samples in RCA Enzyme Mix at  30 °C for  07:00:00

25 Wash samples with 1x PBS twice.

6m

 1 mL 1X PBS for  00:03:00

 1 mL 1X PBS for  00:03:00

rolony crosslinking

26 Add the crosslinking mix to crosslink rolonies with BS(PEG)9. Prepare crosslinking mix.

1h 30m

Component	1x volume (ul)
250mM BS(PEG)9	10
10X PBS	50
ultrapure water	440

Crosslink colonies with BS(PEG)9

🧪 500 μ L 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

imaging

11m

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

10m

Prepare Probe Hybridization Mix

Component	1x volume (ul)
100 uM dcProbe0_AF488	1
100 uM dcProbe0_Cy3	1
100 uM dcProbe0_ATTO647N	1
100% formamide	60
20X SSC	20
ultrapure water	117



add  200 μ L Probe Hybridization Mix to each sample. Incubate for  00:10:00 at  Room temperature

- 28 Wash with washing buffer (10% formamide in 2X SSC, 0.1% TritonX-100) twice. Then, image sample in imaging buffer (10% formamide in 2x SSC buffer).

4m

 1 mL washing buffer for  00:02:00

 1 mL washing buffer for  00:02:00

- 29 Strip with  1 mL 80% formamide in 2X SSC for  00:05:00 .

5m

Wash with  1 mL 2X washing buffer twice.

Repeat the decoding imaging with the next set of decoding probes (dcProbe1, dcProbe2, dcProbe3, dcProbe4, dcProbe5).

- 30 After images of samples stained with dcProbe0, 1, 2, 3, 4, 5 were taken, take the nuclei staining images with Draq5 staining.

14m

add  500 μ L 5 uM DRAQ5 solution to the sample. Incubate for  00:10:00 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