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

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# DART-FISH Protocol

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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 rolony 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 | TheroFischer Scientific | 10977023 |
| Ribonuclease; RNAse H; Conc. 5,000 U/mL; 5,000 U; incl. 10X buffer | Fisher Scientific | 50305945 |

**PROTOCOL integer ID:**
82786

| | Material | Supplier | Catalog Number |
|---|---|---|---|
| | 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 |
| | 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 x 25mm OD No PSA | Grace Bio-Labs | 664304 |
| | Coverslips, Glass, 18mm dia. | Ted Pella | 260369 |
| | Azer Scientific cover glass, No. 1.5, 24 x 60 mm | Neta Scientific | Azer-1152460 |

Probes

| | Oligo name | Vendor | Sequence |
|---|---|---|---|
| | Acr_dc10-Cy5_N9 | IDT | /5AmMC12/CCGATAGTCACGATCTGTGGNNNNNNNN*N |
| | Acr_dc7-488_dT20 | IDT | /5Acryd/CATGGATTCGCGGAGGATCATTTTTTTTTTTTTTTV*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 |

| | Oligo name | Vendor | Sequence |
|---|---|---|---|
| | dcProbe1-AF488 | IDT | /5Alex488N/TCTTGCGTGCGATACGGAGT |
| | dcProbe1-Cy3 | IDT | /5Cy3/AACGGTATTCGGTCGTCATC |
| | dcProbe1-ATTO647N | IDT | /5ATTO647NN/CTGGTTCGGGCGTACCTAAC |
| | dcProbe2-AF488 | IDT | /5Alex488N/AGAACTTGCGCGGATACACG |
| | 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/ |

BEFORE START INSTRUCTIONS

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 ho 6m
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.

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-H2O | 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 37 °C for at least 5 minutes bef 1m 30s 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-H2O | 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 |
| | Superase 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 µL PBSTR quick wash
200 µL 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 µL Acryloyl-X Mix to the sample and incubate for 00:30:00 at Room temperature

quick wash with PBSTR
400 µL PBSTR quick wash

**15** Incubate the sample with 300 µL Acrylamide Solution for 00:30:00 at Room temperat... 30m
.
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 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.

**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 µL 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 µL RNase Digestion Mix
Incubate at 37 °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 mi 3m 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.

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. 6m

1 mL 1X PBS for 00:03:00

1 mL 1X PBS for 00:03:00

## 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 µL 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 µL 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 rolonies 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

## 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 µL 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 imag 14m with Draq5 staining.

add 500 µL 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