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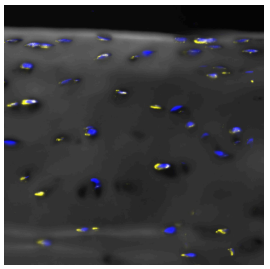
Sequential RNA-FISH on Human Articular Cartilage



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Peter Maye[1,2], Mejeong Lee[1,2], David Rowe[1,2], Martin Lotz[3,2], Merissa Olmer[3,2], dong.shin[4,2]

[1]UConn Health; [2]Human Biomolecular Atlas Project (HuBMAP); [3]Scripps Research; [4]University of Connecticut

Human BioMolecular Atl... UCH Center for Regener... 1 more workspace

Peter Maye
UConn Health

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We use this protocol and it's working

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Abstract

This protocol covers the harvesting, preservation, and implementation of sequential RNA-FISH on human articular cartilage. Distinct elements of this protocol that worked well for human articular cartilage include: (1) the use of Paxgene fixative and stabilizer for the initial preservation of RNA immediately following the initial tissue harvest. (2) the permeabilization of tissue sections for probe access by sequential treatment with a hydrogen peroxide-formamide solution followed by digestion with protease plus, and treatment with HCl. (3) Finally, while the probe design and hybridization chemistry were essentially identical to MERFISH, after covering the tissue in an acrylamide hydrogel, the tissue was not fully cleared by digestion as the loose attachment of chondrocytes sitting inside their lacunae would be lost. Thus, the imaging procedure first captured background matrix autofluorescence, which in the image processing pipeline was background subtracted. Collectively, this protocol worked well on articular cartilage samples derived from 6 different human donors of varying age and ancestry.

Materials

PAXgene Tissue Fix Container (765312, PreAnalytiX)
PAXgene Tissue STABILIZER (765512, PreAnalytiX)
Sucrose (F5-3, Fisher Chemical)
Cryofilm (C-FS 105, SECTION-LAB Co. Ltd. Japan).
PFA (15714, Electron Microscopy Sciences)
PBS (BP3994, Fisher Scientific)
Cryomatrix (6769006, Epredia)
RNAscope Protease Plus (322331, ACD)
Hydrogen Peroxide (H1065, Spectrum)
20xSSC (AM9763, Invitrogen)
Formamide (AM9342, Thermo Fisher)
HCl (SA48-500, Fisher Chemical)
Murine RNase Inhibitor (M0314L, New England Biolabs)
Yeast tRNA (15401-029, invitrogen)
TE Buffer, RNase Free (AM9858, Invitrogen)
Triton-X 100, RNase Free (AC327372500, Thermo Fisher)
Bis-Acrylamide 19:1, 40% (1610144, BioRad)
TEMED (T7024-25ML, Sigma)
Ammonium persulfate (19913-100G, Sigma)
5M NaCl (AM9759, Invitrogen)
1M Tris-HCl (15568025, Thermo Fisher)
Gelslick (50640, Lonza)
Proteinase K (P8107S, New England Biolabs)
0.5M EDTA (AM9260G, Invitrogen)
Dextran Sulfate (S4030, EMD Milipore)

Protocol materials

- Cryofilm **Section-Lab Co. Ltd.** **Catalog #**C-FS 105
- PAXgene Tissue FIX **Qiagen** **Catalog #**765312
- PAXgene Tissue STABILIZER **Qiagen** **Catalog #**765512
- Sucrose **Fisher Scientific** **Catalog #**S5-3
- Tissue-Plus™ O.C.T. Compound **Fisher Scientific** **Catalog #**23-730-571
- 2-Methylbutane **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**M32631
- Paraformaldehyde (PFA) **Electron Microscopy Sciences** **Catalog #**15714
- Phosphate Buffered Saline **Fisher Scientific** **Catalog #**BP3994
- SSC, RNase-free, 20× **Ambion** **Catalog #**AM9763
- Hydrogen Peroxide **Spectrum Chemical MFG Corp** **Catalog #**H1065
- Formamide, deionized, nuclease-free **Ambion** **Catalog #**AM9342
- RNAscope Protease Plus **Avanced Cell Diagnostics** **Catalog #**322331
- HCl **Fisher Scientific** **Catalog #**SA48-500
- Gelslick **Lonza** **Catalog #**50640
- 1M Tris-HCl **Thermo Fisher Scientific** **Catalog #**15568025
- Bis-Acrylamide 19:1, 40% **Bio-Rad Laboratories** **Catalog #**1610144
- 5M Sodium Chloride **Invitrogen** **Catalog #**AM9759
- TE Buffer, RNase Free **Invitrogen** **Catalog #**AM9858
- Triton-X 100, RNase Free **Thermo Fisher Scientific** **Catalog #**AC327372500
- TEMED **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T7024-25ML
- 40mm diameter coverslip **Bioptechs** **Catalog #**40-1313-03193
- Ammonium persulfate **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**19913-100G
- 10% SDS **Invitrogen** **Catalog #**AM9823
- 0.5M EDTA **Invitrogen** **Catalog #**AM9260G
- Proteinase K, Molecular Biology Grade - 2 ml **New England Biolabs** **Catalog #**P8107S
- Dextran Sulfate **Merck Millipore (EMD Millipore)** **Catalog #**S4030
- Yeast tRNA **Invitrogen** **Catalog #**15401-029
- RNase Inhibitor **New England Biolabs** **Catalog #**M0314L

Troubleshooting

Tissue Collection and Preservation

2d

1 Procurement of Medial Femoral Cartilage With Subchondral Bone for FISH: Using an autoclaved 12” hacksaw with 24TPI blade, cut a 3-5mm slab from the middle region of the medial femoral condyle.

2 The posterior (non-weight bearing NWB) and central (weight bearing WB) regions are cut down to maximum 5mmx5mmx5mm pieces, 3 pieces per region.

3 Three pieces of tissue, from one region, are placed in 12mLs of PAXgene Tissue FIX **Qiagen Catalog #**765312 for 24:00:00 on a 4 °C rocker.

1d

4 Pour off fixation solution and replace with PAXgene Tissue STABILIZER **Qiagen Catalog #**765512 and incubate for 24:00:00 at 4 °C on a rocker. Tissue samples can be shipped in PAXgene Stabilizer on On ice and/or stored in the PAXgene stabilizer at -20 °C until ready for tissue processing.

1d

Tissue Embedding

1d

5 Place the sample into 12ml of 30% Sucrose **Fisher Scientific Catalog #**S5-3 at 4 °C for 24:00:00 on a rocker. The 30% sucrose solution was prepared in DEPC-treated water followed by autoclaving.

1d

6 Blot the sample with a kimwipe and place the sample into a cryomold with Tissue-Plus™ O.C.T. Compound **Fisher Scientific Catalog #**23-730-571 .

7 Grasp the cryomold with a pair of forceps and hold at the surface of a container containing 2-Methylbutane **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M32631 prechilled with dry ice. Once completely frozen, store at -80 °C .

Cryosectioning and Section Fixation

2h

8 Cut ~10 micron sections in a cryostat using Cryofilm.
Cryofilm **Section-Lab Co. Ltd. Catalog #**C-FS 105

9 Immediately place tissue sections into the 4% PFA solution. Fix for 01:30:00 at Room temperature . Detach the section from the Cryofilm in the 4% PFA solution in a well-ventilated area, preferably a fume hood, then fix for 00:30:00 more at Room temperature . 2h

Paraformaldehyde (PFA) **Electron Microscopy Sciences Catalog #**15714

Tissue Permeabilization 2h 35m

10 Rinse section in PBS for 00:15:00 at Room temperature . 15m

Phosphate Buffered Saline **Fisher Scientific Catalog #**BP3994

11 Place the section in a solution containing 0.3% Hydrogen Peroxide, 5% Formamide buffered in 0.5x SSC for 01:00:00 at Room temperature . (Warning: when making solution add Formamide to diluted Hydrogen Peroxide. 1h

Hydrogen Peroxide **Spectrum Chemical MFG Corp Catalog #**H1065

Formamide, deionized, nuclease-free **Ambion Catalog #**AM9342

SSC, RNase-free, 20× **Ambion Catalog #**AM9763 Peroxides are potentially explosive)

12 repeat step 10 15m

13 Add 50 ul of RNAscope Protease Plus on the section and incubate for 00:30:00 at 37 °C 30m

RNAscope Protease Plus **Avanced Cell Diagnostics Catalog #**322331

14 Transfer the section into 0.1 N HCl solution and incubate for 00:05:00 at Room temperature 5m

HCl **Fisher Scientific Catalog #**SA48-500

15 Transfer section to 2x SSC and incubate for 00:30:00 at Room temperature . 30m

SSC, RNase-free, 20× **Ambion Catalog #**AM9763

Probe Synthesis and Preparation for Hybridization

12m

16 **Encoding Probe Design**: Probe Dealer Software created by Dr. Siyuan Wang's lab was used to design encoding probes. This software is a user friendly Matlab Program the runs with a graphical user interface. Instructions on how to use it and download it are freely available from Dr. Siyuan Wang's Lab
https://campuspress.yale.edu/wanglab/probedealer/

17 **Encoding Probe Synthesis**: Probes were synthesized at IDT in a 96-well plate format 25nmole synthesis scale. The number of oligonucleotides designed against each gene is variable and dependent on transcript length and sequence similarity to other genes. We try and have a minimum of 50 oligos pooled together for each gene. Ideally, more is better as the signal-to-noise ratio improves. For smaller transcripts, where the oligo pool is under 50 oligos, we have duplicated readout probe target sites for better detection.

Well Position	Name	Sequence
A1	ACAN_ENST00000559004	GACACTCAGCGAGTTGTCATGGTCTGAAGTTTAGGAGGAGGGTAATGATAGA
A2	ACAN_ENST00000559004	CTGGAAGGTGAACTTCTCTGGAGATGTTGCTTAGGAGGAGGGTAATGATAGA
A3	ACAN_ENST00000559004	TCTGGGATGTCCACAAAGTCTTCACCTGTGTTAGGAGGAGGGTAATGATAGA
A4	ACAN_ENST00000559004	GGTATAGGCTGGTTCCCATTCTGTCTGGTTTTAGGAGGAGGGTAATGATAGA
A5	ACAN_ENST00000559004	GGGCAGTAGTCTCTAGAACTTCTCCAGAAGTTAGGAGGAGGGTAATGATAGA
A6	ACAN_ENST00000559004	CGCAGAGGTAGAAGTCTCTAGAACTTCTCCTTAGGAGGAGGGTAATGATAGA
A7	ACAN_ENST00000559004	CCACTGATGTCCTCTACTCCAGAGACAGAATTAGGAGGAGGGTAATGATAGA
A8	ACAN_ENST00000559004	AGAGGTCTCTAGACCTTCTCCTGAAGGAAGTTAGGAGGAGGGTAATGATAGA
A9	ACAN_ENST00000559004	GGAAACAGTTGGGAATCCAGATGGAAGTCCTTAGGAGGAGGGTAATGATAGA

blue: 30bp antisense sequence, red: complementary sequence to readout probe

18 **Readout Probe Synthesis, Resuspension, and Storage**: Cy5 and Alexa750 disulfide conjugated readout probes were ordered from Biosynthesis (www.biosyn.com). Probes were resuspended in TE Buffer containing 0.1% v/v Triton X-100 at a concentration of [M] 100 micromolar (µM) and stored -20 °C . Probes were aliquoted into multiple tubes to prevent repeated freeze-thawing to extend life. The sequence information for readout probes was previously detailed in https://doi.org/10.1073/pnas.1612826113.

TE Buffer, RNase Free **Invitrogen Catalog #**AM9858

Triton-X 100, RNase Free **Thermo Fisher Scientific Catalog #**AC327372500

19 **Anchor Probe Synthesis, Resuspension, and Storage**: An anchor probe was ordered from IDT, resuspending in RNase-free TE buffer at a concentration of [M] 100 micromolar (µM), at stored at -20 °C . The anchor probe targets the polyA mRNA tails and crosslinks the mRNA to the acrylamide gel through the 5' Acrydite modification. T+ identifies the locked nucleic acid modification to increase binding affinity. \5Acryd\TTGAGTGGATGGAGTGTAATT+TT+TT+TT+TT+TT+TT+TT+TT+TT+T
IDT Ordered: 100 nmole synthesis scale, RNase-Free, HPLC Purified.

TE Buffer, RNase Free **Invitrogen** **Catalog #**AM9858

20 **Encoding Probe Preparation**: Each encoding probe is resuspended into a final concentration of [M] 500 micromolar (µM) with RNase-free TE buffer. (For a 25nmole synthesis this would be 50ul). Then oligos designed against each gene are pooled together. (500um x 50ul)/(50 oligos x 50ul/oligo)=10uM per oligo within the pool. This is essentially a 2000x concentration to make a 5nM amount per oligo for experiments. This is sufficiently concentrated to mix one gene probe pool with many other gene probe pools for your studies.

TE Buffer, RNase Free **Invitrogen** **Catalog #**AM9858

21 Prepare 25 µL of encoding probe at [M] 10 nanomolar (nM) per oligo for one section in 30% Formamide and 10% Dextran Sulfate in 2xSSC. 1m

SSC, RNase-free, 20× **Ambion** **Catalog #**AM9763

Formamide, deionized, nuclease-free **Ambion** **Catalog #**AM9342

Dextran Sulfate **Merck Millipore (EMD Millipore)** **Catalog #**S4030

22 Heat at 80 °C for 00:10:00, then cool down. 10m

23 Prepare 25 ul of 2 uM of Anchor probe in 2x SSC containing 2% RNase inhibitor, 0.2% tRNA, 10% Dextran Sulfate, and 30% Formamide. 1m

SSC, RNase-free, 20× **Ambion** **Catalog #**AM9763

Formamide, deionized, nuclease-free **Ambion** **Catalog #**AM9342

RNase Inhibitor **New England Biolabs** **Catalog #**M0314L

Yeast tRNA **Invitrogen** **Catalog #**15401-029

Dextran Sulfate **Merck Millipore (EMD Millipore)** **Catalog #**S4030

24 Combine probe pool with anchor probe mix. (This is a sufficient amount of encoding probe for one tissue section. Scale up as desired).

Hybridization

25 Transfer the section into 30% Formamide in 2xSSC solution and incubate at room temperature for 15 minutes.

SSC, RNase-free, 20× **Ambion Catalog #**AM9763

Formamide, deionized, nuclease-free **Ambion Catalog #**AM9342

26 Hybridize the section with encoding probe solution for 42:00:00 at 37 °C

1d 18h

27 Wash section in 30% Formamide in 2xSSC solution for 00:30:00 at 37 °C and repeat wash 3-5 times.

30m

Gel Embedding Tissue Section

30m

28 Transfer the section into a 4% acrylamide solution containing 300 mM NaCl and 50 mM Tris solution. Let it incubate for 00:30:00 at Room temperature .

30m

Bis-Acrylamide 19:1, 40% **Bio-Rad Laboratories Catalog #**1610144

5M Sodium Chloride **Invitrogen Catalog #**AM9759

1M Tris-HCl **Thermo Fisher Scientific Catalog #**15568025

29 Place a 10 ul drop of 4% acrylamide solution containing 300mM NaCl, 50mM Tris, 0.03% APS and 0.15% TEMED in the center of bind-silane functionalized 40mm round coverslip.

Bis-Acrylamide 19:1, 40% **Bio-Rad Laboratories Catalog #**1610144

5M Sodium Chloride **Invitrogen Catalog #**AM9759

Ammonium persulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**19913-100G

TEMED **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T7024-25ML

1M Tris-HCl **Thermo Fisher Scientific Catalog #**15568025

40mm diameter coverslip **Bioptechs Catalog #**40-1313-03193

30 Immediately transfer the section onto the drop of acrylamide.

31 Place a Gelslick-functionalized coverslip (size 22×22mm) gently over the section to sandwich the droplet, being careful not to create bubbles.

Gelslick **Lonza Catalog #**50640

32 Push the Gelslick-functionalized coverslip gently to make a thin gel. Remove the extra gel solution.

33 Allow polymerization to happen for 01:00:00 at Room temperature . 1h

34 Remove the Gelslick-functionalized coverslip from the section

35 Add 65 ul of 4% acrylamide solution in 300mM NaCl, 50mM Tris with 0.03% APS and 0.15% TEMED onto the tissue section.

36 Place the Gelslick-functionalized slide (size 75×50mm) gently over the slide to sandwich the droplet, being careful not to create bubbles

37 Allow the polymerization to happen for 01:00:00 at Room temperature . 1h

38 Remove the Gelslick-functionalized slide from the section

Tissue Digestion, Imaging, and Sequential Hybridization of Readout Probes 1h 30m

39 Prepare tissue digestion solution (50 mM Tris, 300mM NaCl, 1mM EDTA, 0.5% Triton-X100, 1% SDS, Proteinase K (200ug/ml)) and add 3ml per 40mm round coverslip. Incubate the for 01:00:00 at 37 °C 1h

1M Tris-HCl **Thermo Fisher Scientific Catalog #**15568025

Triton-X 100, RNase Free **Thermo Fisher Scientific Catalog #**AC327372500

5M Sodium Chloride **Invitrogen Catalog #**AM9759

0.5M EDTA **Invitrogen Catalog #**AM9260G

10% SDS **Invitrogen Catalog #**AM9823

Proteinase K, Molecular Biology Grade - 2 ml **New England Biolabs Catalog #**P8107S

40mm diameter coverslip **Bioptechs Catalog #**40-1313-03193

40 Thoroughly rinse sections for 00:30:00 in 2xSSC at Room temperature . 30m

Repeat wash 5 times.

SSC, RNase-free, 20× **Ambion Catalog #**AM9763

41 From this point forward the tissue section is ready for sequential rounds of readout probe hybridization and imaging. For human articular cartilage, tissue digestion did not fully clear the tissue. Therefore, we imaged the matrix background under every channel and subtracted it from readout probe rounds of imaging. Additionally, because the matrix was easily detectable, we used it in replace of fiduciary beads for image registration. Additional details of MERFISH protocols can be found at https://moffittlab.github.io/.

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