Histology Core Learning Lunch:

Single Cell Insights: RNA-FISH Techniques in Musculoskeletal tissues

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University of Pennsylvania

Guest Speakers:

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University of Pennsylvania

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PENN
CENTER for
MUSCULOSKELETAL
DISORDERS

Monday October 28, 2024

Overall Aims

- The mission of the Penn Center for Musculoskeletal Disorders Histology Core is to provide comprehensive, high quality histology services to musculoskeletal researchers at the University of Pennsylvania and the broader research community.

- The Specific Aims of the core are:
  - To provide guidance and training on the capabilities, advantages, and disadvantages of the various methodologies to assess musculoskeletal tissue structure and composition through formal educational enrichment programs and one-on-one interactions.
  - To provide expertise and service for histological and histomorphometric assays of musculoskeletal tissues.
  - To develop new histologically-based techniques that will be applicable to musculoskeletal research.
  - To provide funding for development of new projects and collaborations and to develop preliminary and/or feasibility data for investigators.

Facilities and Equipment

Paraffin

- Tissue processing
- 4 x paraffin microtomes
- Embedding station

Frozen

- 3 x cryostat microtomes
- Cryofilm technique for undecalcified sectioning

***Plastics**

- Processing and embedding
- Polycut microtome
- Transition

Facilities and Equipment

Imaging and Analysis

- Double headed teaching microscope
- *Brightfield and fluorescence microscopes with high resolution cameras for image capture
- Slide scanner (Axio Scan.Z1, full service only)

Services Offered

Self Service

- Virtual training is required for using core equipment (email Dr. Arroyo or access the self service website under “Online training” for training videos: https://www.med.upenn.edu/pcmd/histologycore-selfservice).
- Users can perform:
  - Paraffin embedding
  - Paraffin sectioning
  - *Plastic sectioning
  - Frozen sectioning (additional training for cryofilm)
  - Microscopy and imaging (bright field and fluorescence)
- Dr. Arroyo is available to supervise and assist.

*Please talk to us re: Plastic sectioning station

Services Offered

Consumables included in self-service:

- Paraffin
- OCT
- Cassettes
- OCT and Paraffin Molds
- Nitrile Gloves

For an additional fee:

- Cryofilm – Please log in to the Histology Core Self Service website to purchase cryofilms:

https://www.med.upenn.edu/pcmd/histologycore-selfservice

- Slide boxes – you can BYO
- Users must provide chucks, blades and slides

Services Offered

Full Service – Complete Project Delivery

- Free consultation, protocol development, pilot projects*
- Project completed by core technician
  - Paraffin processing and embedding (~1 week)
  - Paraffin sectioning (~2 weeks)
  - Frozen sectioning (cryofilm) (~2 weeks)
  - Routine histochemical staining (~2 weeks)
  - Slide scanning (~1 week)
  - Immunohistochemistry (~2 weeks)
  - Fluorescence *in situ* hybridization - FISH (~2 weeks)

- Accelerated service for urgent projects ($+30%).
- Budget preparation: Projects costing more than $100 require PI approval.

Services Offered

Sample Submission

Histology Core Service Request Form

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Please Fill Out Applicable Info

Place Labeled Samples on Tissue Processor

Send Form to arroyoe@pennmedicine.upenn.edu and leave a copy next to your samples

Name: _____ ***Email:*** _____

Project Description (1-2 sentences describing project goals - required unless paraffin processing only):

_____

_____

Species: _____ ***Tissue:*** _____ ***P.I.:*** _____

P.I. Email: _____ ***Drop-Off Date:*** _____ ***Date Needed:*** _____

****Samples for Paraffin Processing must be fixed e.g. in formalin, decalcified (if desired), rinsed, put in 70%EtOH prior to drop off***

Paraffin

Processing:

Tissue Processor

☐ Yes # of samples: _____

Processor Cycle (if known):

Sectioning & Staining

Sample No.	Section Thickness (um)	# of sections per slide	# of Slides	Stain Type [if desired]

Services Offered

Usage Charges

- Free consultation and protocol development
- Very competitive charges for full and self service histology
- Generous subsidies offered for Penn users

Feedback

- Online surveys
- Ideas for new services

Thank you for using our core!

- **Acknowledging the Histology Core in Publications**

For publications that describe work that benefited from the Histology Core, please include a statement similar to:

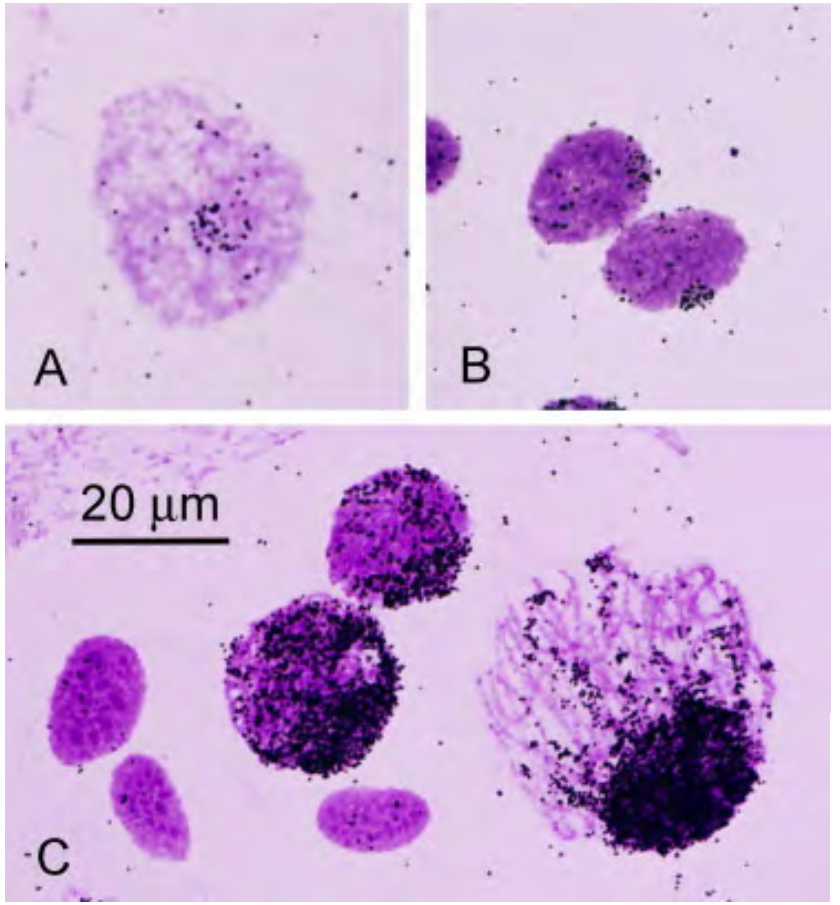
“This study was supported by the Penn Center for Musculoskeletal Disorders Histology Core (P30 AR069619)”.

https://www.med.upenn.edu/pcmd/histologycore-homepage.html

In situ Hybridization (*Is*H)

- Developed in 1969 by Joseph G. Gall and Mary Lou Pardue
- Generated tritiated ribosomal RNA(rRNA) by applying ^{3}H-uridine to cultured oocytes
- Identified the molecular hybrid formation between tritiated rRNA and intact DNA in *Xenopus* oocytes preparations in the meiotic nucleus
- Very interesting and easy read of Methods article by Joseph G. Gall (2016).

Methods 97(2016):4



Genetics 63(1969):378

Gall & Pardue, *Genetics 63(1969):378*
as "…reimaged" by J. G. Gall for *Methods 97(2016):4*

*Is*H evolved to F*ISH**

Isotopic ISH

- Uses radioactive isotopes to generate RNA/DNA probes.
- Uses autoradiographic emulsion to detect probes RNA/DNAs binding *in situ*.
- Typically uses a dark room
- Takes time (about a month)

Non-Isotopic ISH

- RNAScope (Tyramide Peroxidase amplification) or Molecular Instruments (Hybridization Chain Reaction) to amplify signal, among others.
- Uses fluorescence or HRP conjugated bound probes to detect in situ binding.
- Can detect multiple probes
- Takes two to three days for whole procedure detection in tissues

*Fluorescent in situ hybridization

FISHing with the PCMD Histology Core

2. You provide: fixed tissues, probes to be tested, and a filled-out service request form

3. We perform RNA-FISH on the tissues provided, scan results and provide you with slides and imaging data.

The Core's Free Expert Advice

Provided by you

Fixed Tissues

Buy Probe(s)

Service Request Form

Process & Embed Fixed Tissues

OCT/Cryofilm & Paraffin Sectioning

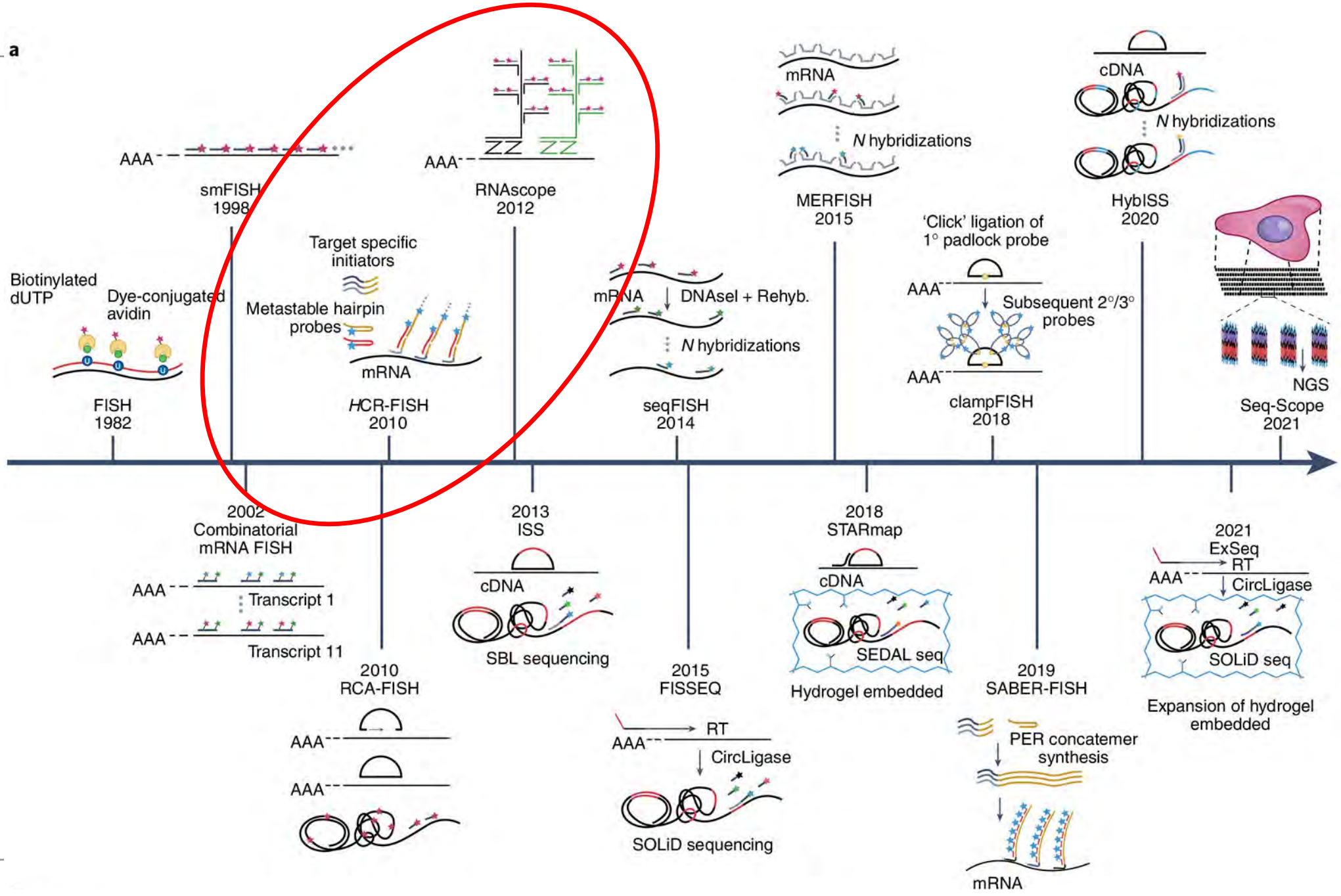
FISH

Histology Core Provides

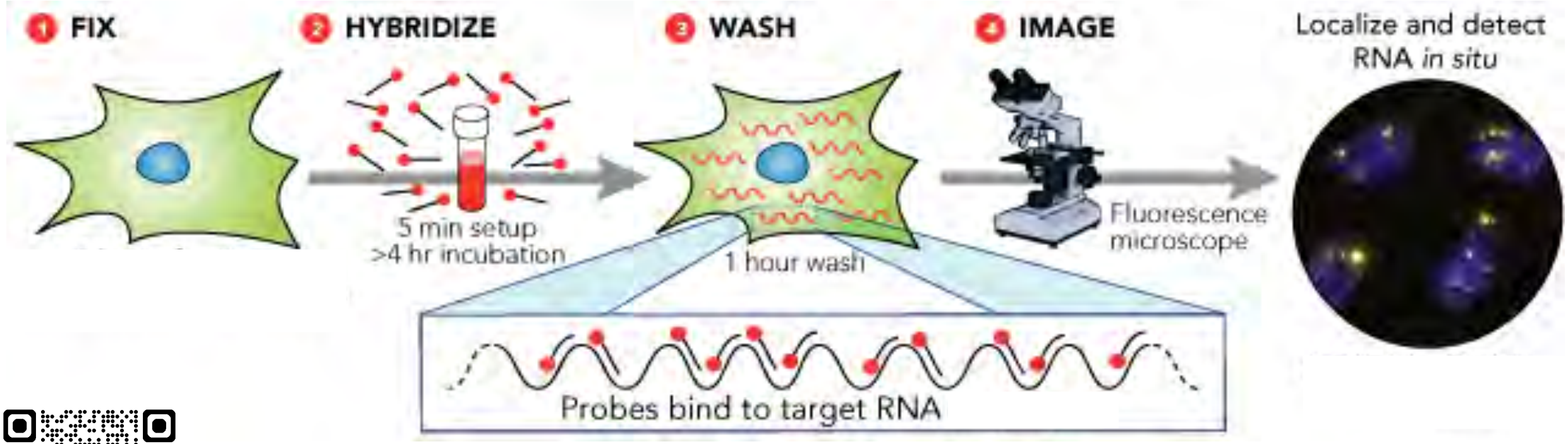
Observe/Scan Results

1. You conceive an idea for an RNA-FISH and may consult the core *free* for protocol development.

4. Use your obtained data in manuscripts, and to obtain more funding. Please acknowledge **P30 AR069619** in your writing.


a
Biotinylated dUTP
Dye-conjugated avidin
FISH 1982
AAA
smFISH 1998
Target specific initiators
Metastable hairpin probes
mRNA
HCR-FISH 2010
AAA
RNAscope 2012
mRNA
DNAsel + Rehyb.
N hybridizations
seqFISH 2014
mRNA
N hybridizations
MERFISH 2015
'Click' ligation of 1° padlock probe
AAA
Subsequent 2°/3° probes
AAA
clampFISH 2018
cDNA
N hybridizations
HybISS 2020
NGS
Seq-Scope 2021
2002 Combinatorial mRNA FISH
AAA
Transcript 1
AAA
Transcript 11
2010 RCA-FISH
AAA
AAA
2013 ISS
cDNA
SBL sequencing
2015 FISSEQ
AAA
RT
CircLigase
SOLiD sequencing
2018 STARmap
cDNA
SEDAL seq
Hydrogel embedded
2019 SABER-FISH
PER concatemer synthesis
mRNA
2021 ExSeq
AAA
RT
CircLigase
SOLiD seq
Expansion of hydrogel embedded

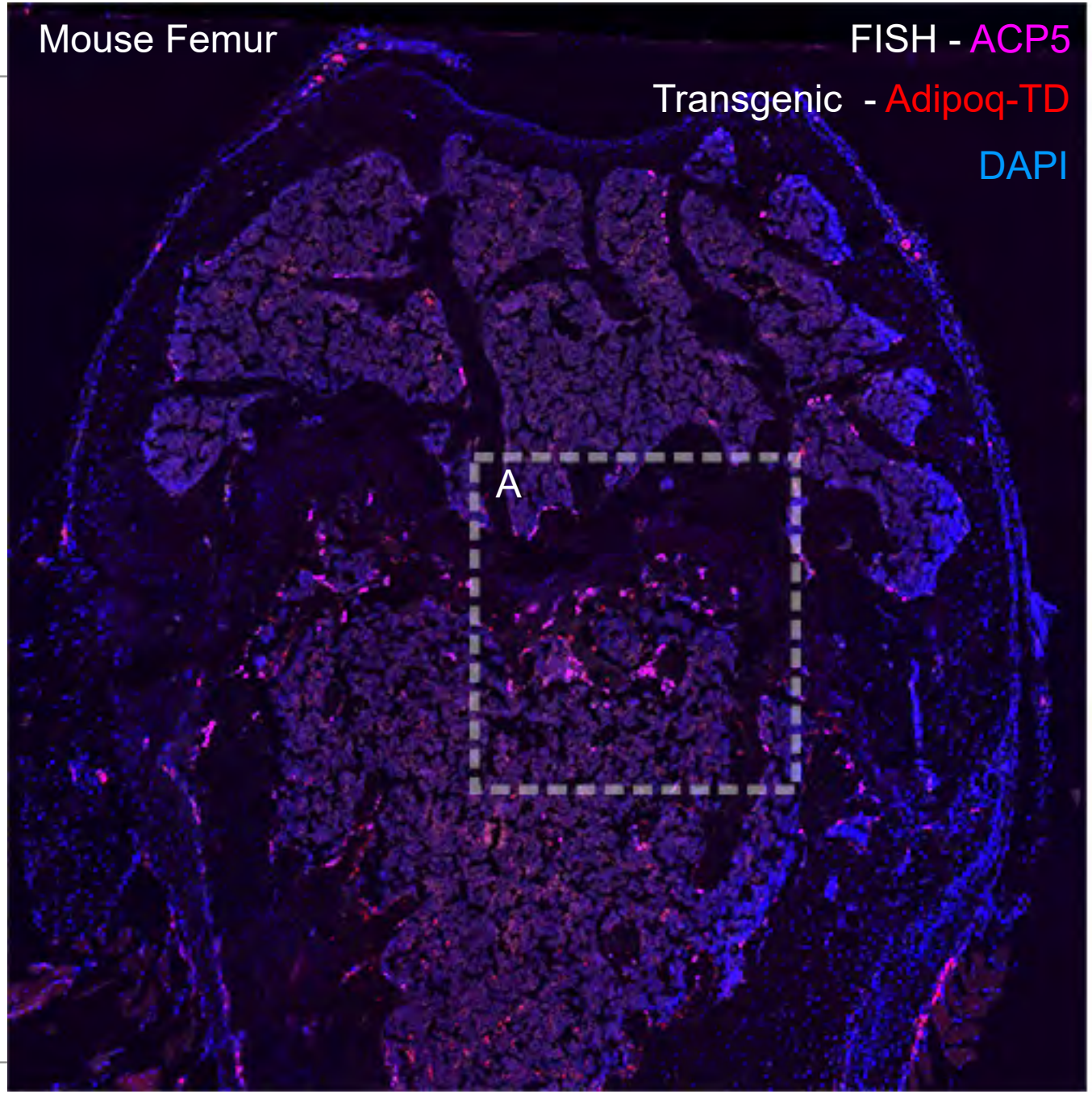
FISH basic steps

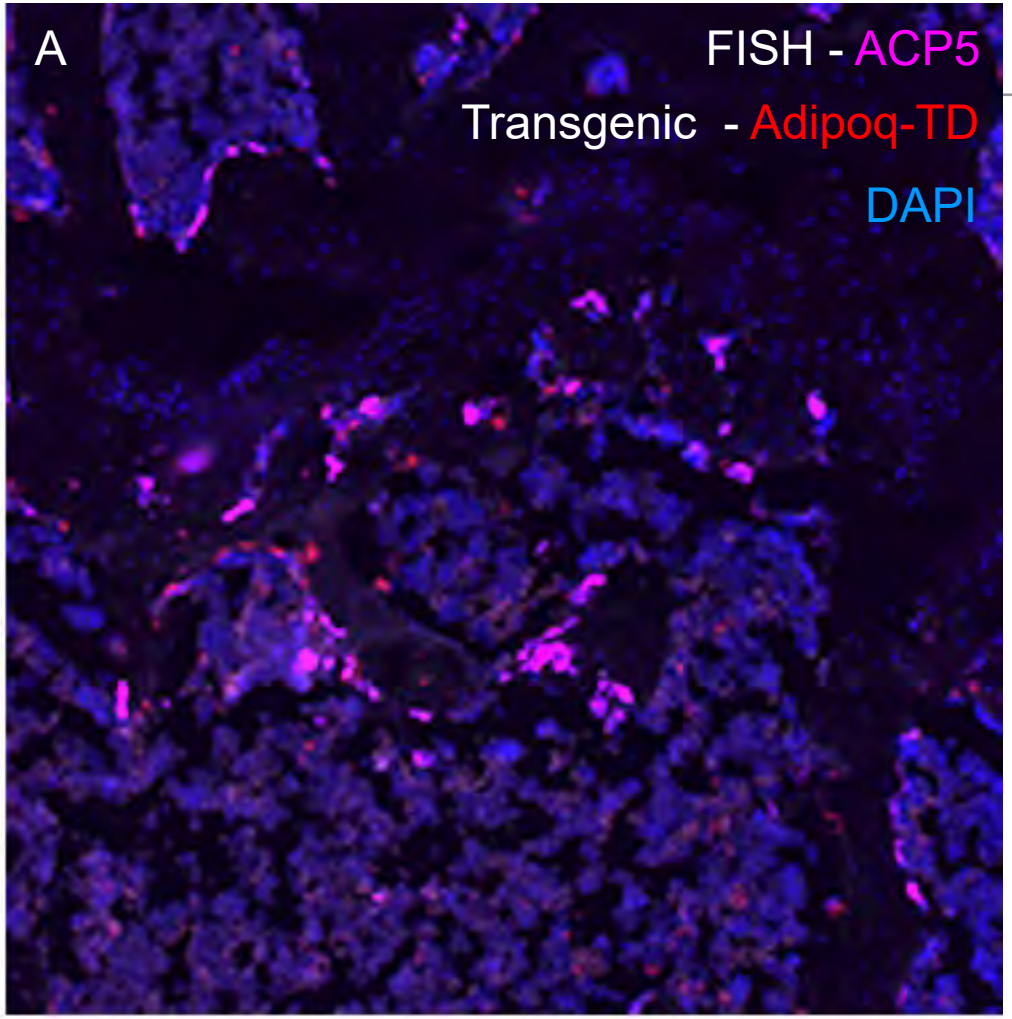


Other FISH Reviews:

PeerJ. 2020 Mar 19;8:e8806

Nature Cell Bio ***24***, *815–824 (2022)*

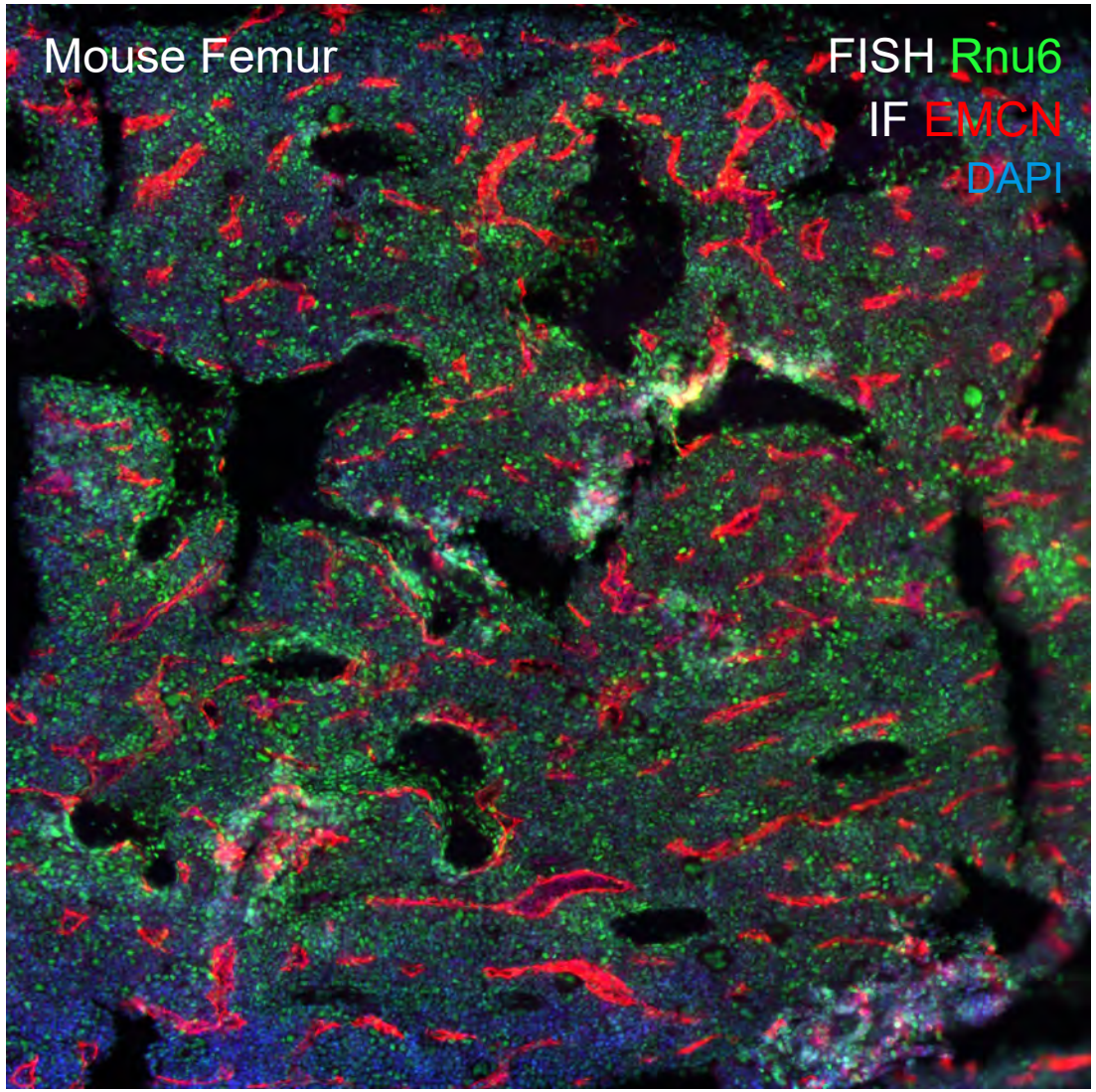




HCR-FISH

Courtesy of Sung Kim

PCMD Histology Core



PCMD Histology Core

HCR – FISH with Immunofluorescence

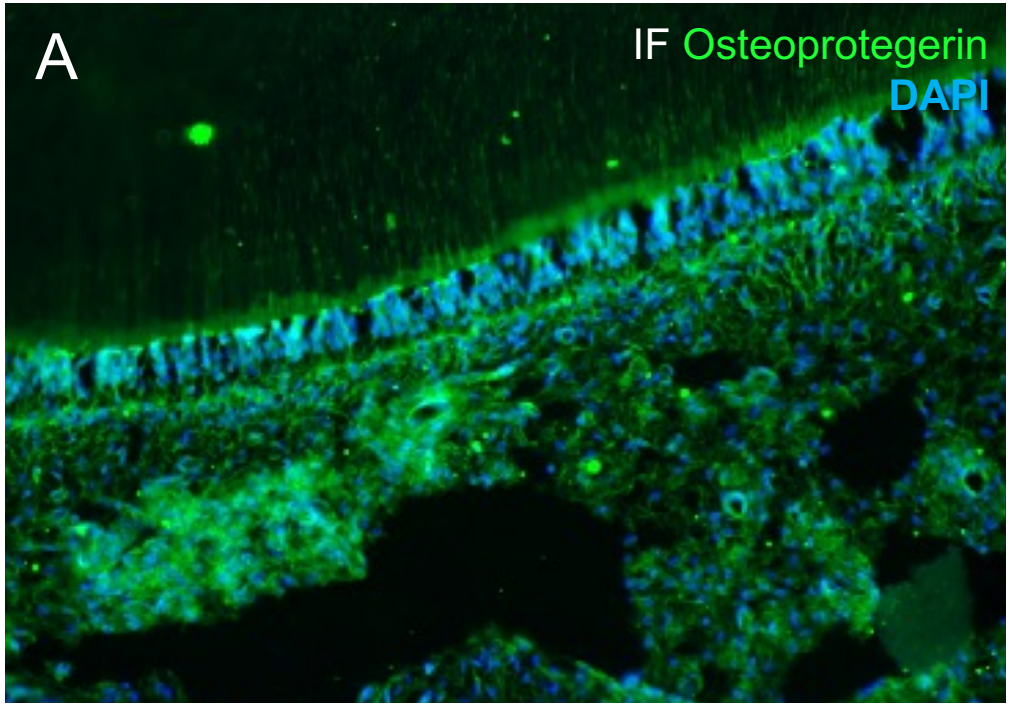
EMCN — endomucin
Rnu6 — Small nuclear RNA - 6

We can also provide IF with HCR-RNA FISH!

Please contact us!

Immunofluorescence in Cryofilm Sections

Courtesy of Aleksandra Dwornicka



PCMD Histology Core

Our personnel

QR Code website

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Technical Director
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At your Service…

PCMD Histology Core Learning Lunch Series

HCR™ RNA-FISH staining and imaging

Qi He

Oct. 28th, 2024

Outline

- Introduction
- Sample preparation
- Sample sectioning
- HCR™ RNA-FISH staining
- Imaging

HCR™ RNA-FISH staining

First introduce the concept of hybridization chain reaction (HCR) in 2004

By 2010, HCR began to be used in RNA detection, especially in combination with FISH technology, and the concept of HCR RNA FISH came into being.

FISH is a classic technique for RNA in situ hybridization, where fluorescently labeled probes hybridize to target RNA molecules for detection. However, traditional FISH often has limited signal strength, especially when detecting low-abundance RNA.

By integrating HCR with FISH, researchers could amplify the fluorescent signals through a chain reaction, achieving high sensitivity and single-molecule resolution in RNA detection.

Advantages of HCR RNA FISH:

- Enzyme-free amplification
- High sensitivity
- Low background noise
- Multiplex staining

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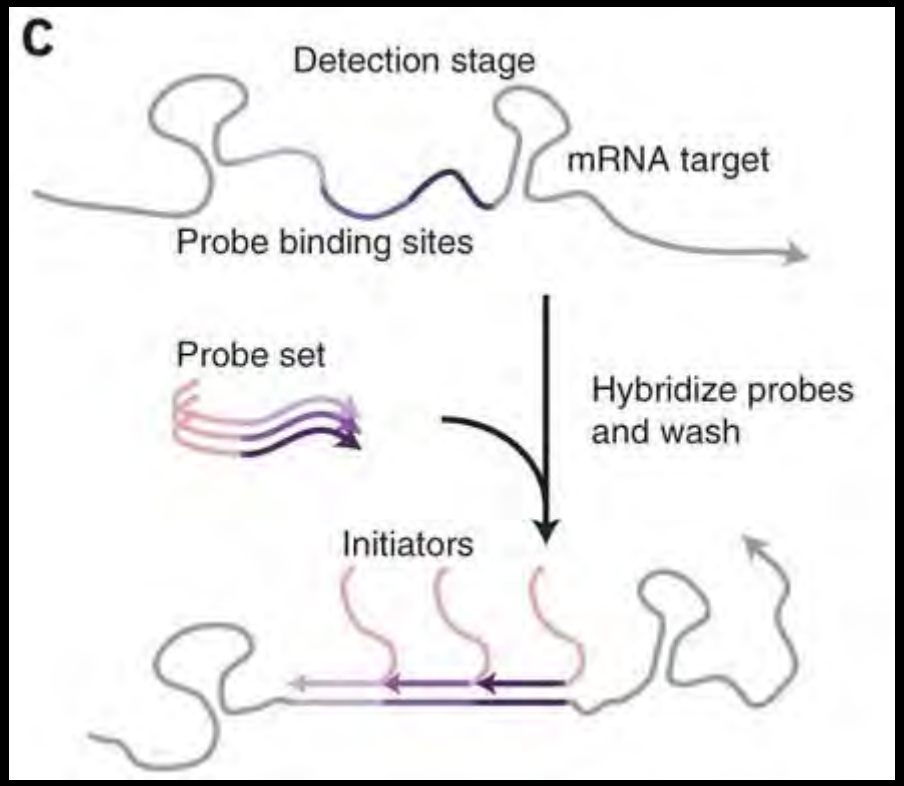
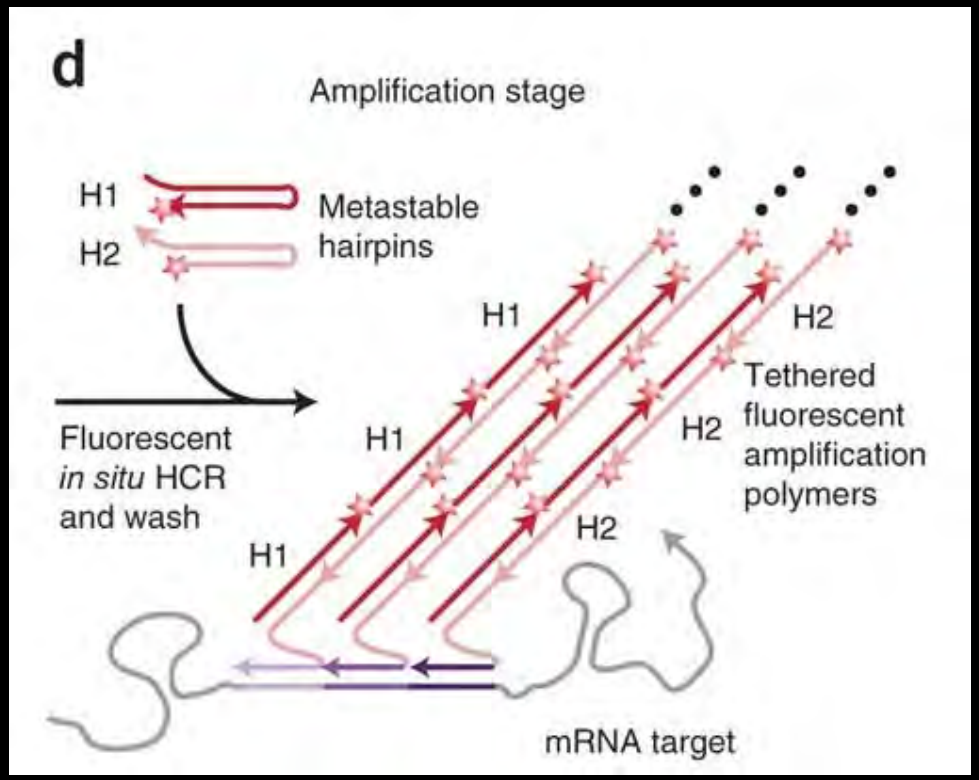
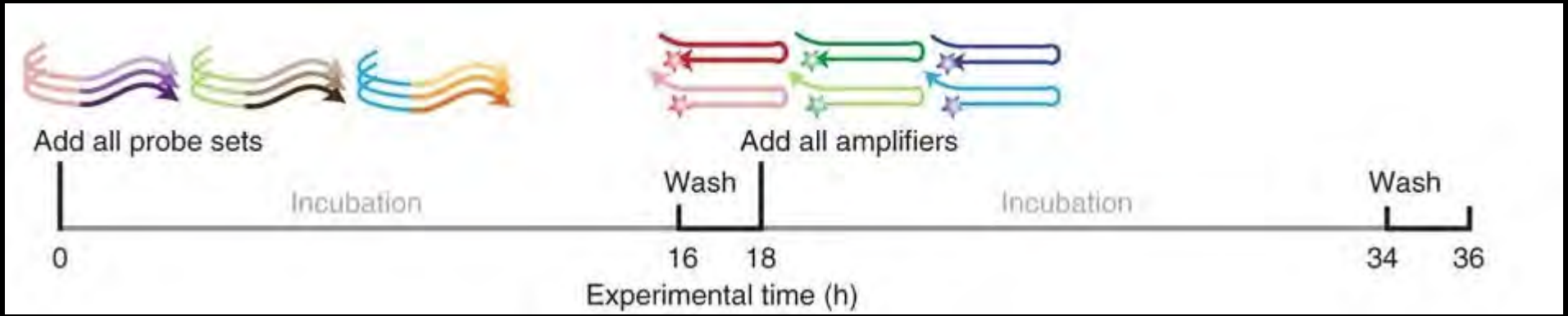
How It Works


c
Detection stage
mRNA target
Probe binding sites
Probe set
Hybridize probes
and wash
Initiators


d
Amplification stage
H1
H2
Metastable
hairpins
Fluorescent
in situ HCR
and wash
H1
H2
Tethered
fluorescent
amplification
polymers
H1
H2
H1
H2
mRNA target

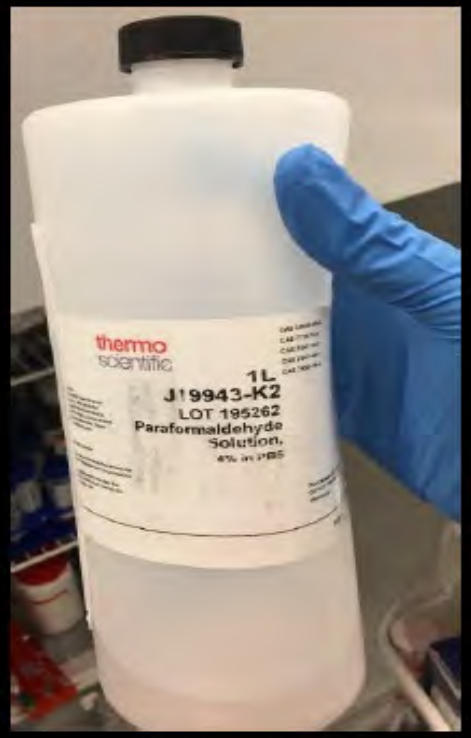

Add all probe sets
Add all amplifiers
Incubation
Wash
Incubation
Wash
0
16
18
34
36
Experimental time (h)

Frozen Sample preparation

Chemicals:

RNaseZap™

4% Paraformaldehyde (PFA)



Sucrose

UltraPure™ DNase/RNase-Free Distilled Water

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Frozen Bone tissue preparation

Cleaning: Clean the dissection tools such as scissors and forceps using RNAseZap.

Fixation: Freshly dissected bone tissues were immediately placed in ice-cold 4% PFA solution and fixed at 4℃ for 24 hours.

Dehydration: After fixation, wash sample in DNase/RNase-Free Distilled Water 3 times and then immersed into 30% sucrose overnight at 4℃.

Embedding: Finally, the tissues are embedded in OCT.

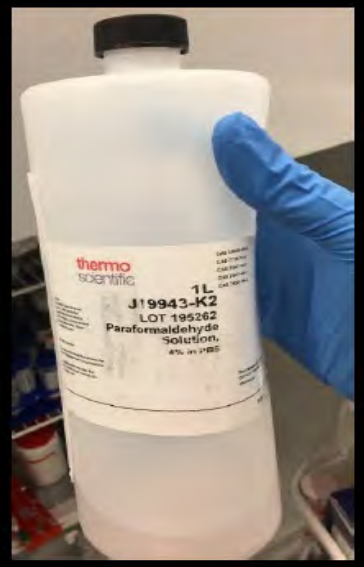
Preparation time: 2 days

Paraffin Sample preparation

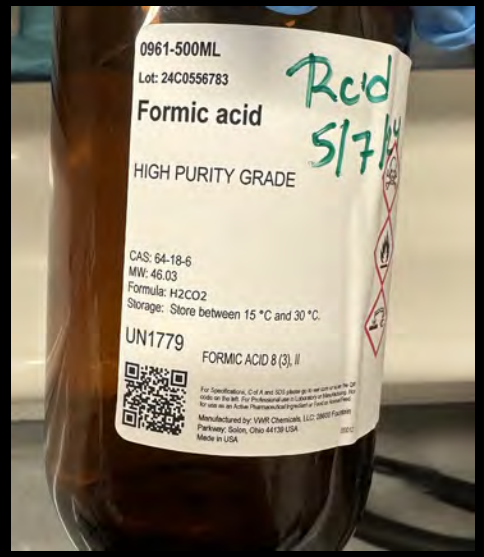
Chemicals:

RNaseZap™

4% (PFA)



Formic acid



Sodium citrate

UltraPure™ DNase/RNase-Free Distilled Water

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Paraffin Bone tissue preparation

Cleaning: Clean the dissection tools such as scissors and forceps using RNAseZap.

Fixation: Freshly dissected bone tissues were immediately placed in ice-cold 4% PFA solution and fixed at 4℃ for 24 hours.

Decalcification: After fixation, wash sample in DNase/RNase-Free Distilled Water. Fill the bottles with Morse's solution (10% sodium citrate and 22.5% formic acid) [1] (about 30 ml per sample). Allow samples to decalcify at room temperature for 48 h.

Processing: Using the processing service of histology core

Embedding: Embed the tissues in paraffin blocks using a standard method.

Preparation time: more than 3 days

[1] de Charleroy C, Haseeb A, Lefebvre V. Preparation of Adult Mouse Skeletal Tissue Sections for RNA In Situ Hybridization. Methods Mol Biol. 2021;2245:85-92.

Tips for Paraffin Bone tissue preparation

Morse's solution: Must be prepared with distilled water and prepare no more than 3 days before use.

After fixation with 4% PFA, the samples can be stored in 70% alcohol for 7-10 days

Decalcification time using Morse solution depends on mouse age and tissue size, Generally, the femur or tibia of mice within 10-20 weeks only takes 48 hours, and the joints take 72 hours.

Processing should be done within 1-2 days after decalcification. It needs to be stored in 70% alcohol.

The water in the water bath needs to be DNase/RNase-Free distilled water.

Sectioning

Superfrost Plus Slide

Section thickness: 8μm (Frozen) or 6μm (Paraffin)

Paraffin: Dry the slides at 45 ℃ on a cleaned slide warmer until the sections have fully spread (about 2 h), and then dry them overnight at room temperature on a clean surface covered with 2–3 layers of Kimwipes in a protected location

Section should be stored at -20℃ (Frozen) or 4℃ (Paraffin)

Staining

HCR™ RNA-FISH protocol for frozen sections

5. Prepare probe solution by adding 0.4 pmol of each probe set (e.g. 0.4 μL of 1 μM stock) to 100 μL of probe hybridization buffer at 37 °C.
   NOTE: *For single-molecule RNA imaging, use higher probe concentration (e.g., 16 nM) to increase probe hybridization yield. If desired, this approach can also be used to increase signal for subcellular quantitative RNA imaging.*

Prepare probe solution by adding 1.6 pmol of each probe set (e.g. 1.6 µL of 1 µM stock) to 100 µL of probe hybridization buffer at 37℃.

8. Place a coverslip on the sample and incubate overnight (>12 h) in the 37 °C humidified chamber.

7. Place a coverslip on the sample and incubate overnight (>12 h) in a dark humidified chamber at room temperature.
   NOTE: *For single-molecule RNA imaging, amplify for a shorter period of time to ensure single-molecule dots are diffraction-limited.*

Note: Place coverslip

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Staining

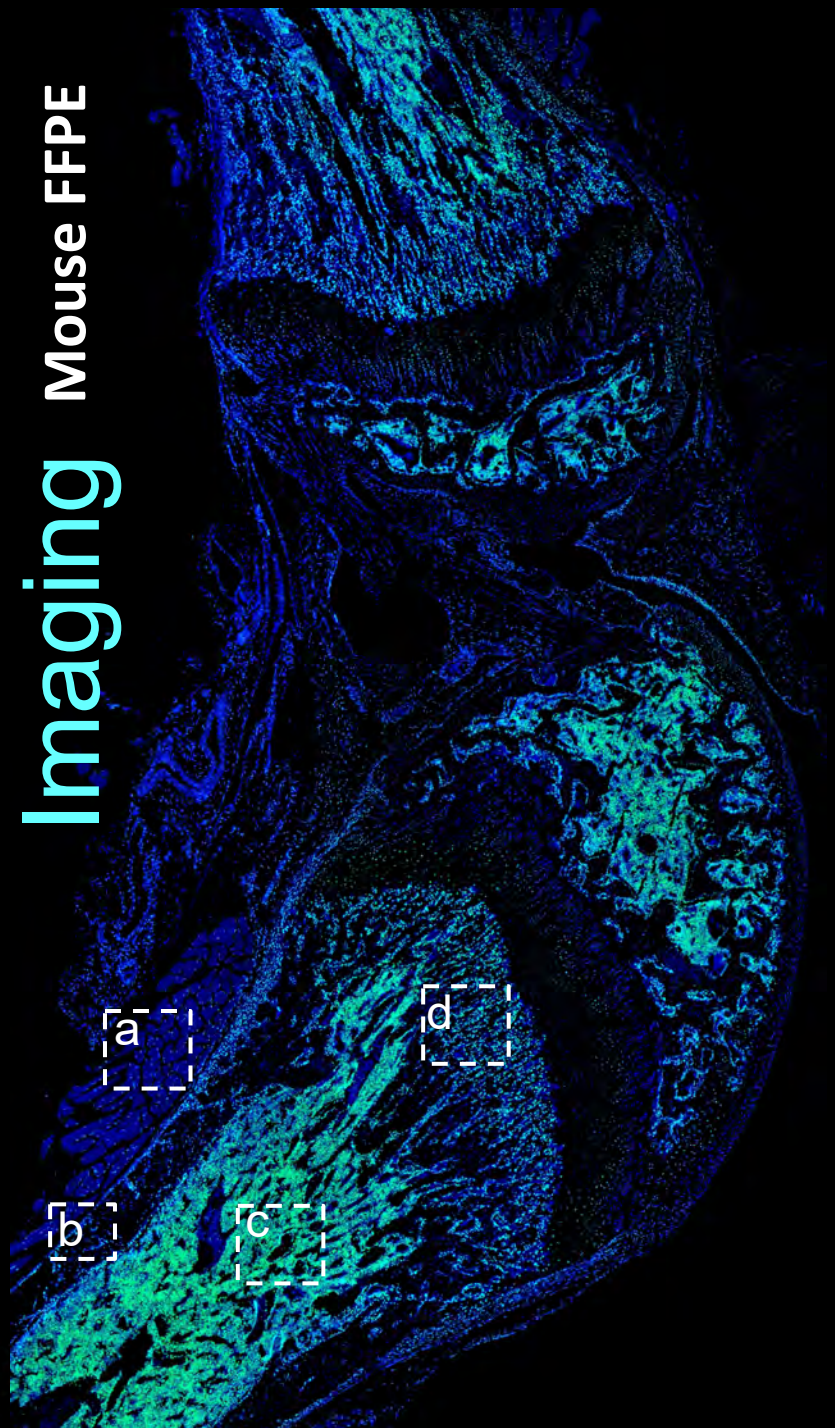
HCR™ RNA-FISH protocol for FFPE tissue sections

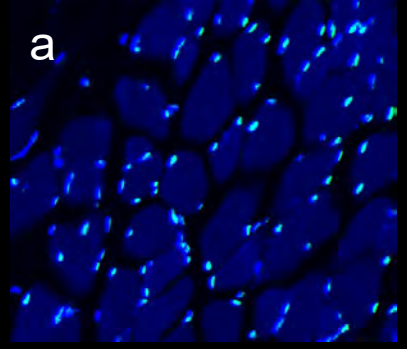
5. Bring 500 mL of 1× Tris-EDTA buffer (pH 9.0) in a beaker to boil in a microwave.
   NOTE: *1× citrate buffer (pH 6.0) can be used in place of Tris-EDTA buffer (pH 9.0).*
6. Maintain Tris-EDTA buffer temperature at 95 °C on a hot plate.
7. Immerse slides for 15 min.
8. Remove beaker from hot plate and add 100 mL of nanopure water every 5 min to allow temperature to decrease to 45 °C in 20 min.
9. Immerse slides in 400 mL of nanopure water in a separate container for 10 min at room temperature.
10. Immerse slides in 1× PBST for 2 × 2 min at room temperature.
    NOTE: *avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the tissue.*
11. Dry slide using a Kimwipe. Avoid touching the tissue.
12. Draw a barrier around the tissue using a hydrophobic pen.

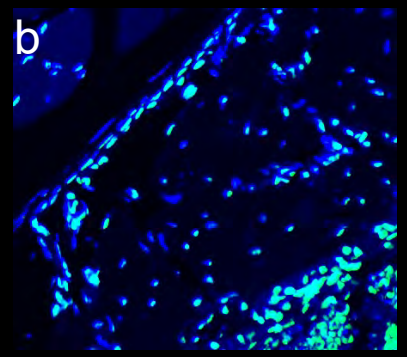
Delete the above steps and start directly from step 13. This is because the high temperature in this step will destroy the morphology of the paraffin sections, and directly using Proteinase K will not affect the results.

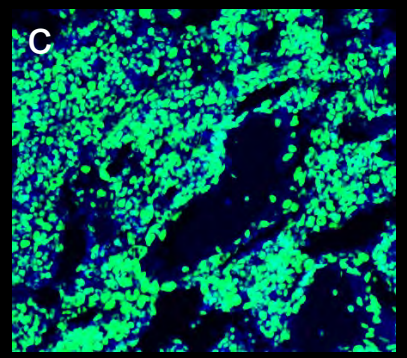
Imaging

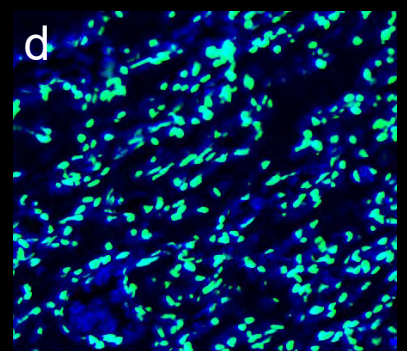
Mouse FFPE



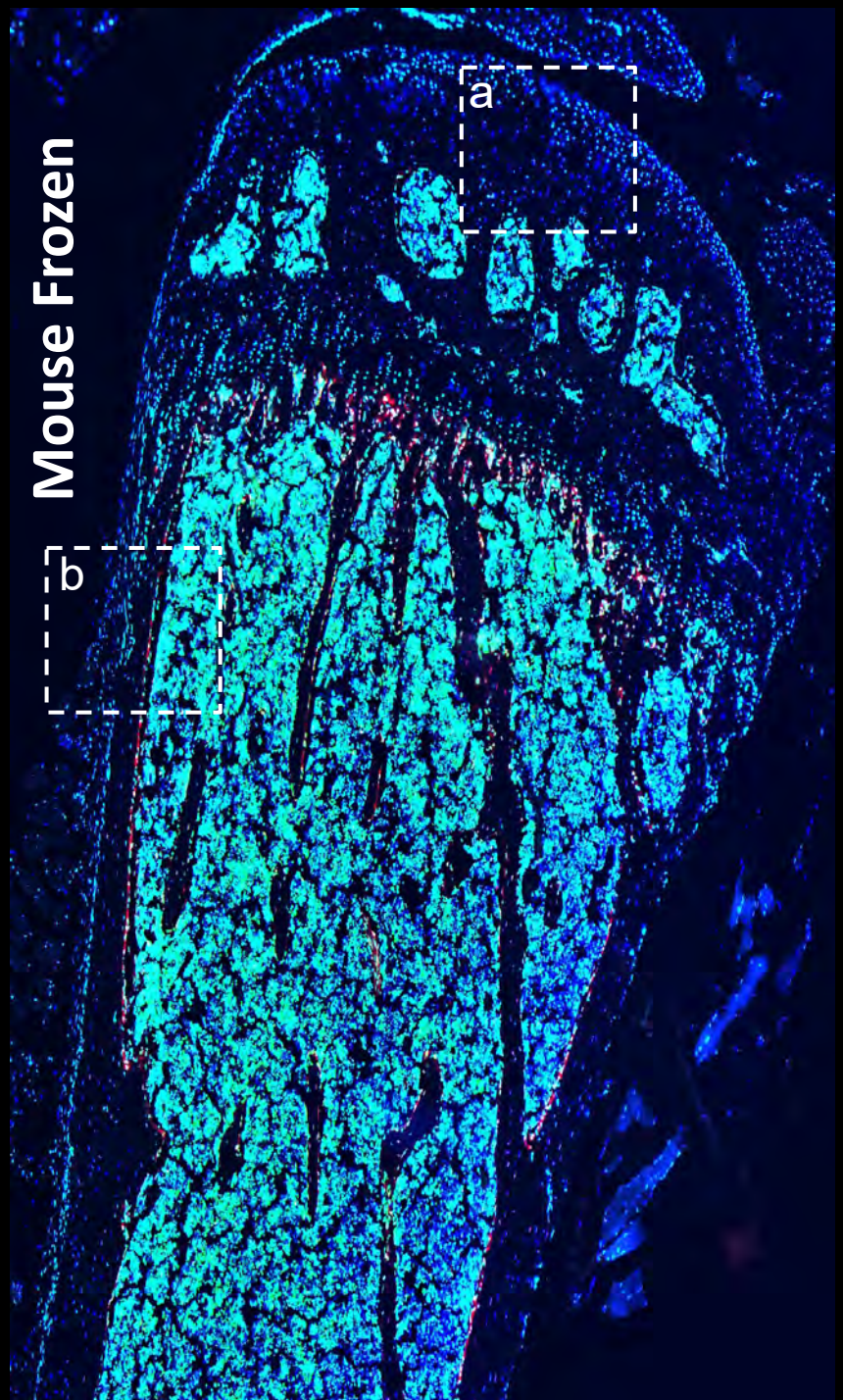


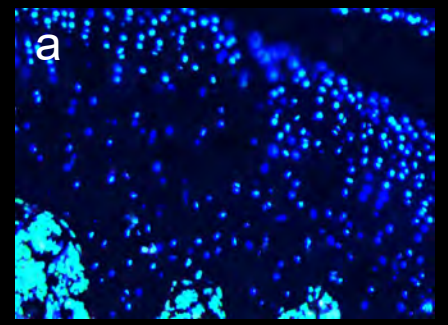


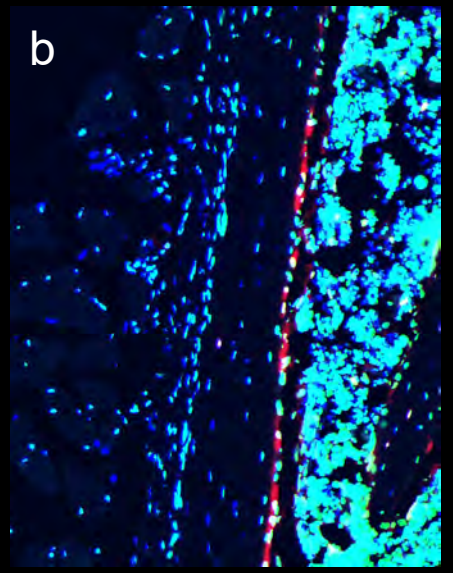




Mouse Frozen



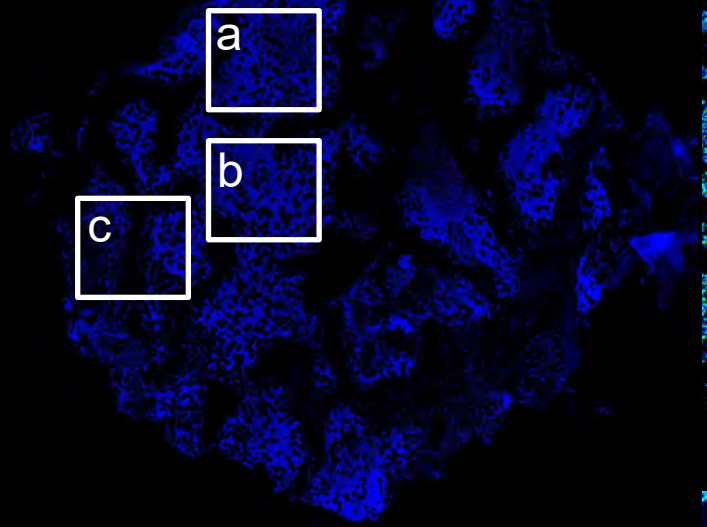


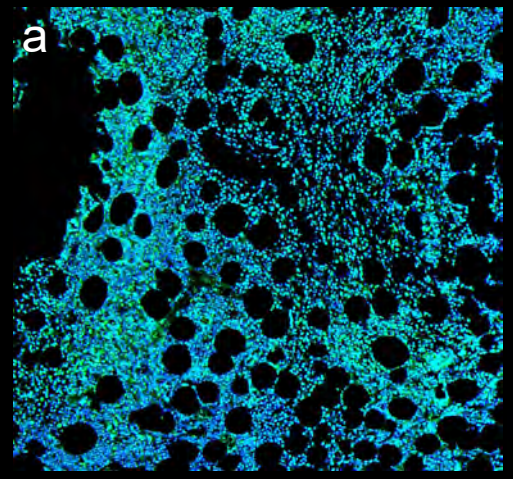


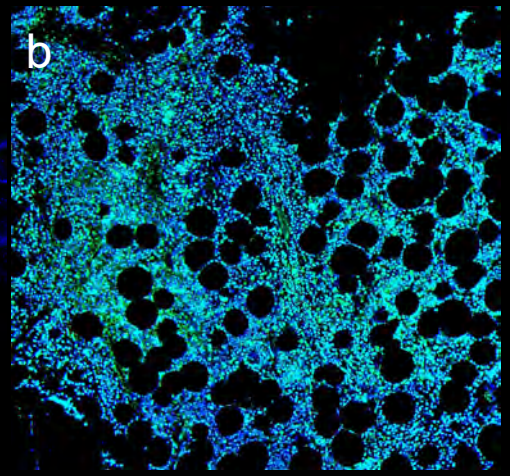
Imaging

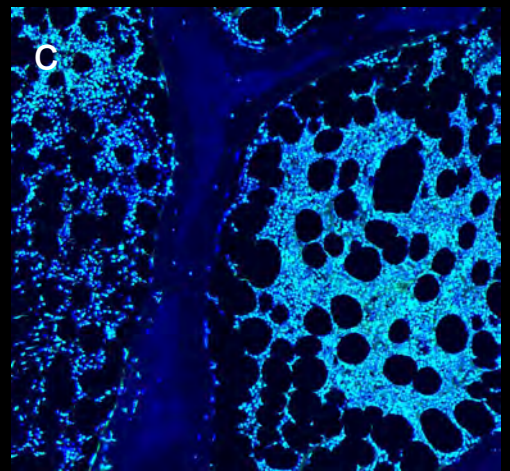
Human FFPE

DAPI/ Rnu6



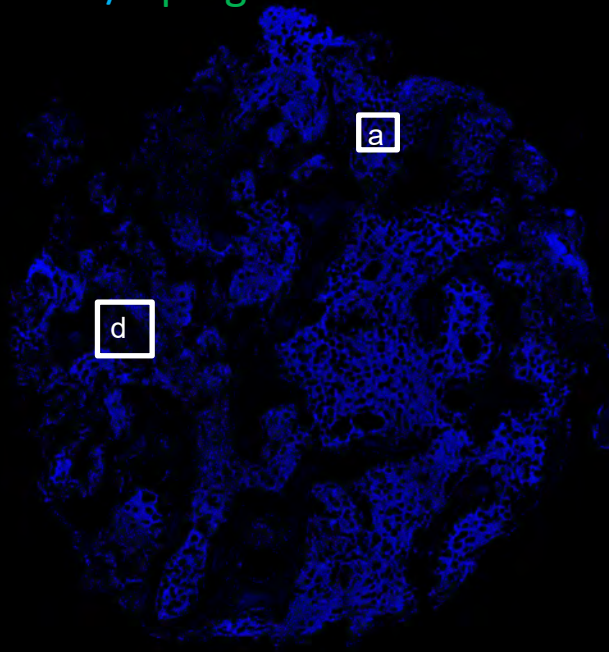


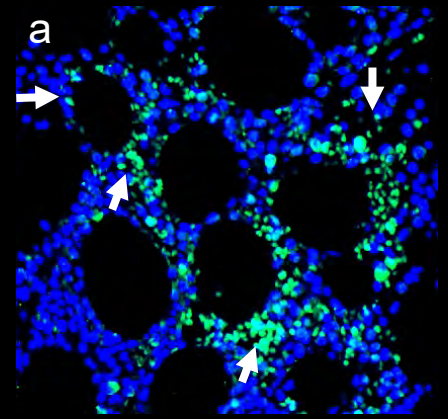


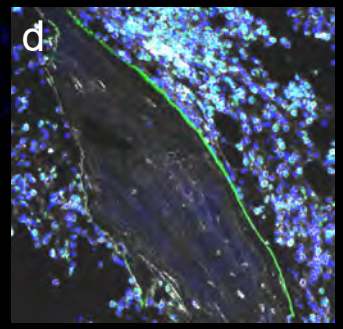


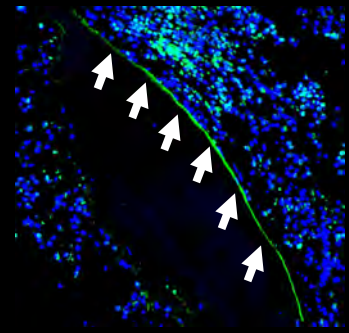
Human FFPE

DAPI/ Pparg



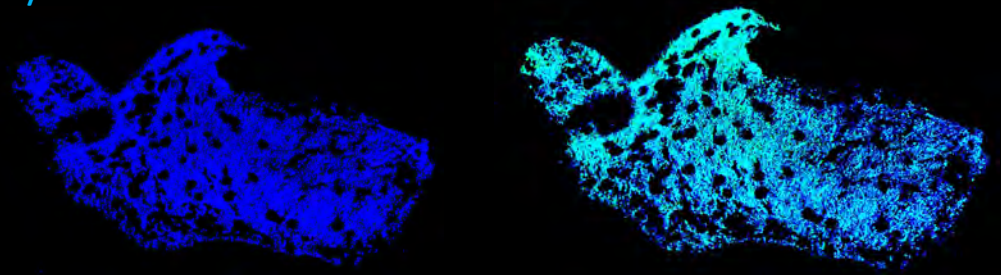






Monkey FFPE

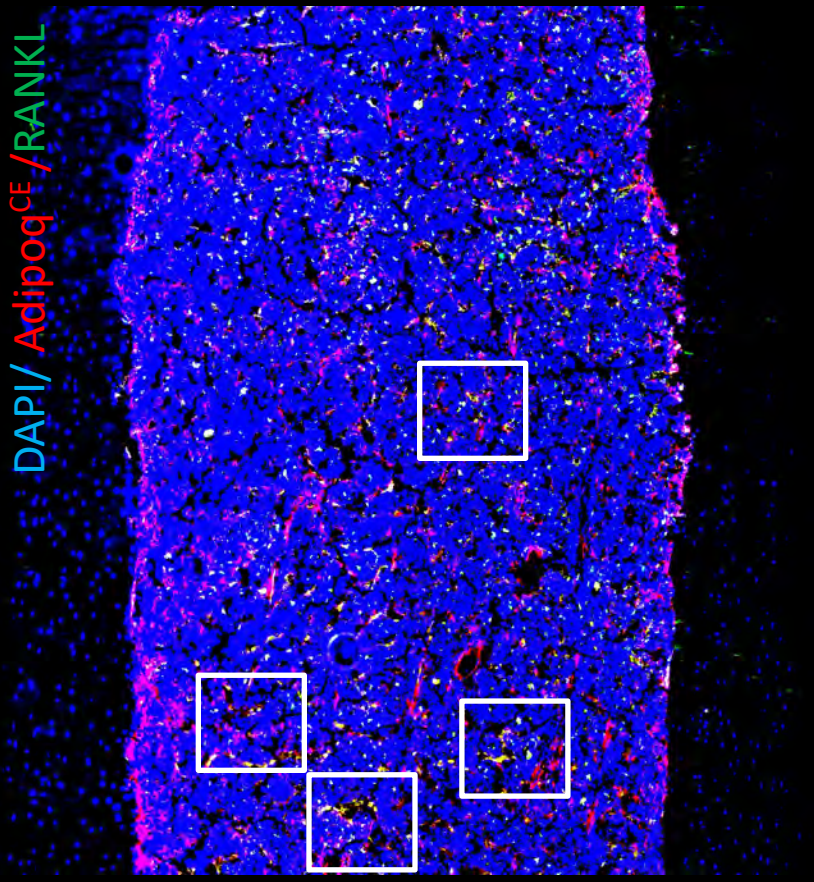
DAPI/ Rnu6

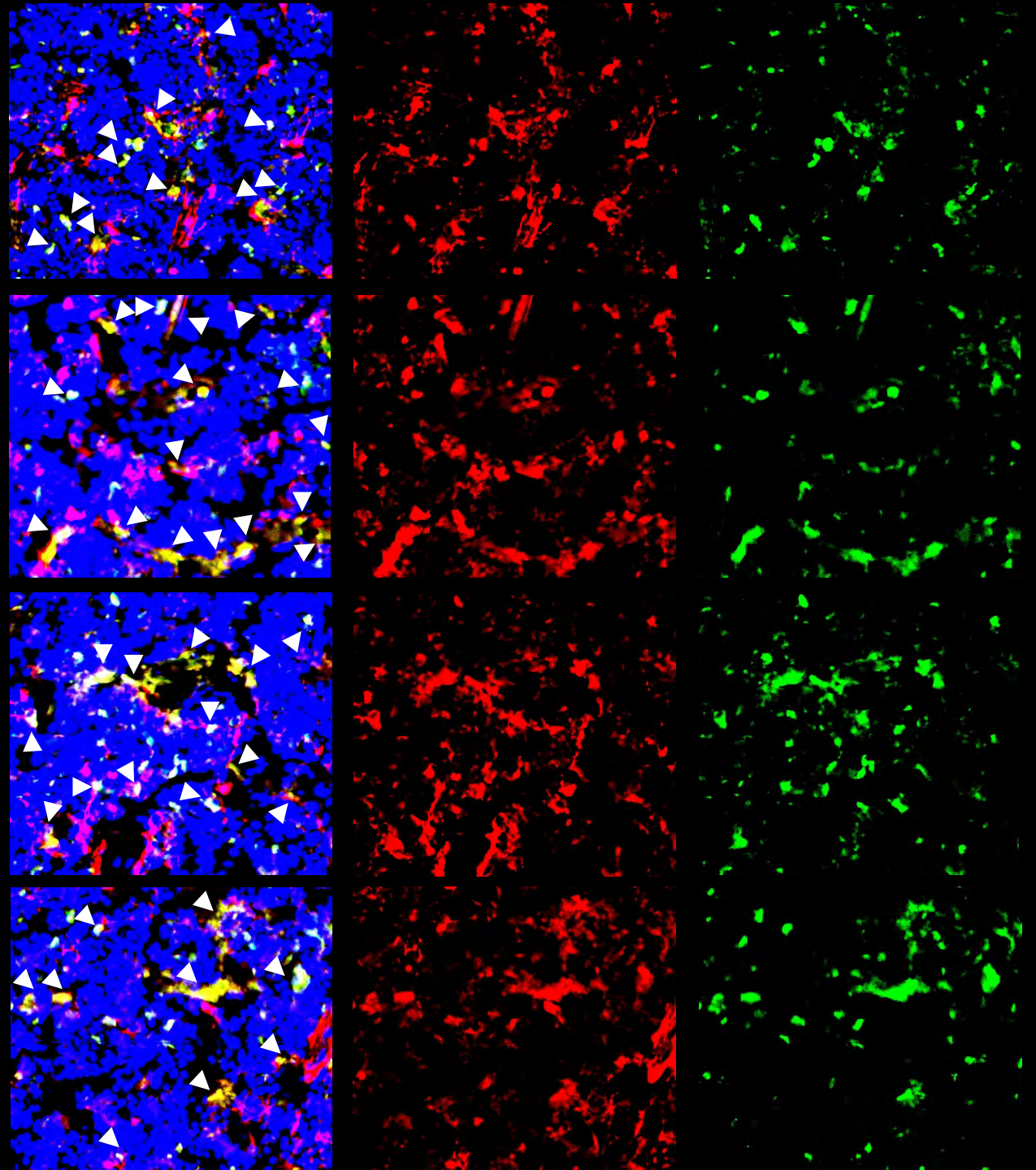


Penn

Imaging

Mouse Frozen

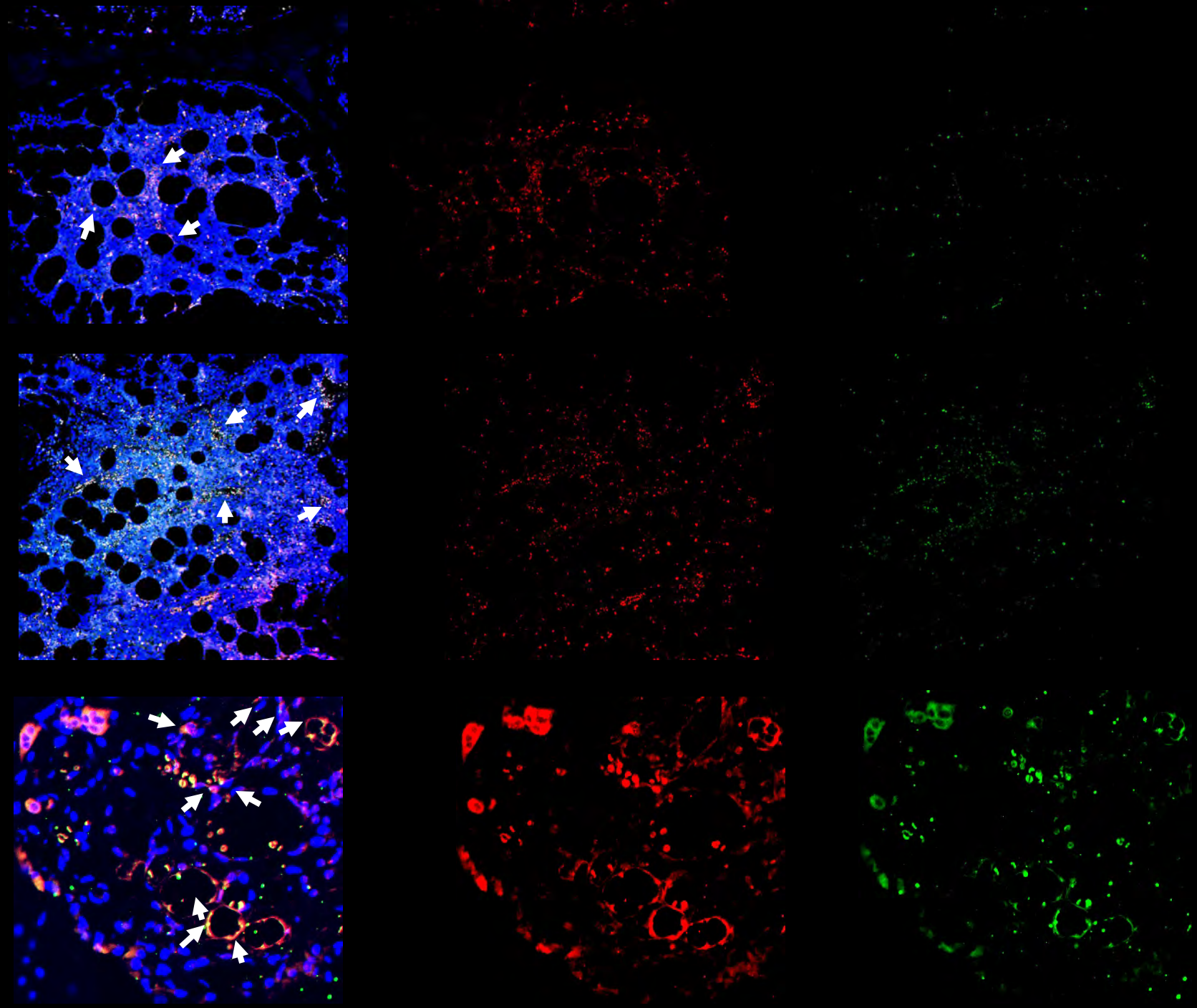




Imaging

Human FFPE

DAPI/Pparg/Rankl



Thank you for listening

RNAscope assays for skeletal tissues

Marco Angelozzi

Staff Scientist – Lefebvre Lab

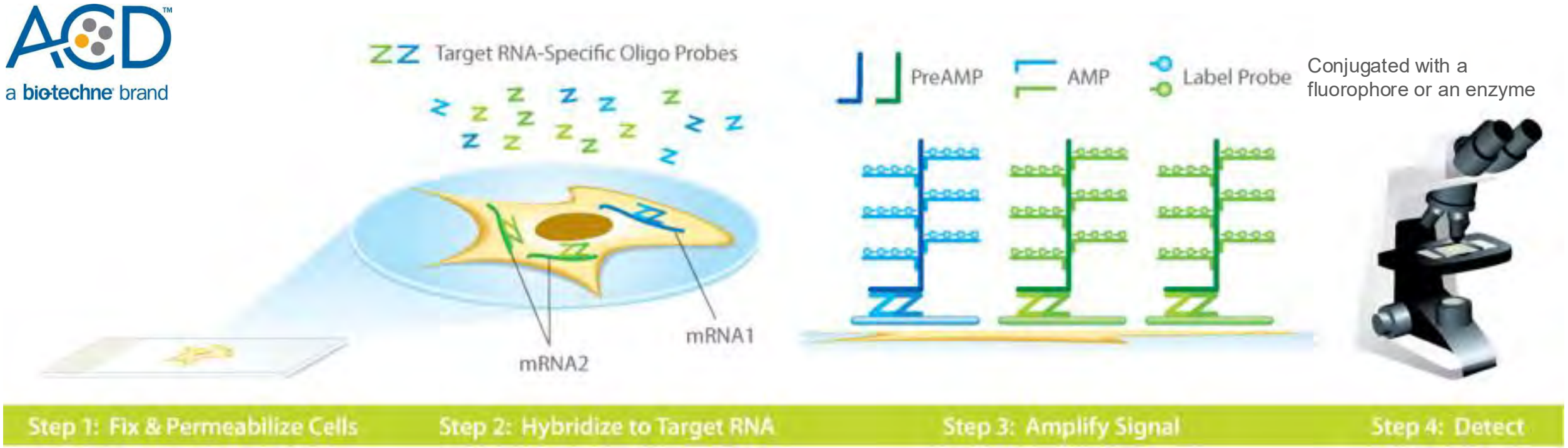
Children's Hospital of Philadelphia

PCMD Histology Core – Learning Lunch
October 28th, 2024

The RNAscope technology

RNAscope is a one-day RNA in situ hybridization (ISH) assay developed by **Advanced Cell Diagnostics** (ACD) in 2012.

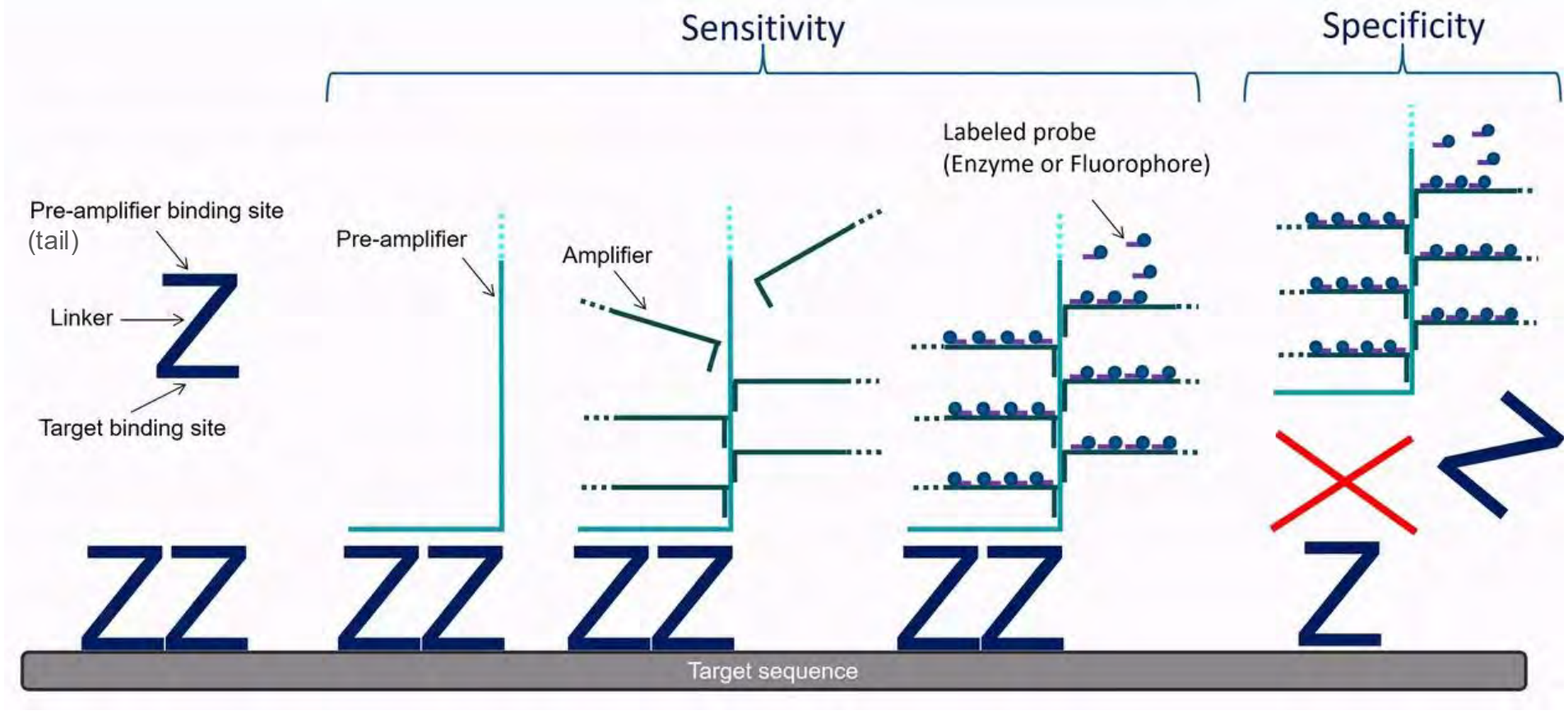
Compatible with **FFPE**, fresh frozen or fixed frozen tissues.



(*Wang et al., J Mol Diagn. 2012*)

RNAscope probes

- RNAscope uses **double-Z probes** containing 18 to 25 bases complementary to target RNA, a spacer sequence and a 14-base tail sequence.
- Two tails form a 28-base hybridization site for the pre-amplifier (increased specificity).
- ~20 probe pairs are used to target a ~1 kb region of the target RNA.



RNAscope available assays

- RNAscope assays:
  - **Chromogenic Single Assay** (**red** or brown)
  - Chromogenic Duplex Assay (red & green)
  - Fluorescent MultiPlex Assay (up to 4 targets)
  - Fluorescent HiPlex Assay (up to 12 targets in FFPE and 48 in frozen samples)

- Other assays:
  - BaseScope Assay → detection of one nucleotide differences in RNA targets (e.g., gene editing, SNVs)
  - miRNAscope Assay → detection of small RNAs (e.g., miRNAs, siRNAs, ASOs)
  - DNAscope Duplex Assay → for DNA ISH (e.g., gene copy number variations, gene rearrangement)

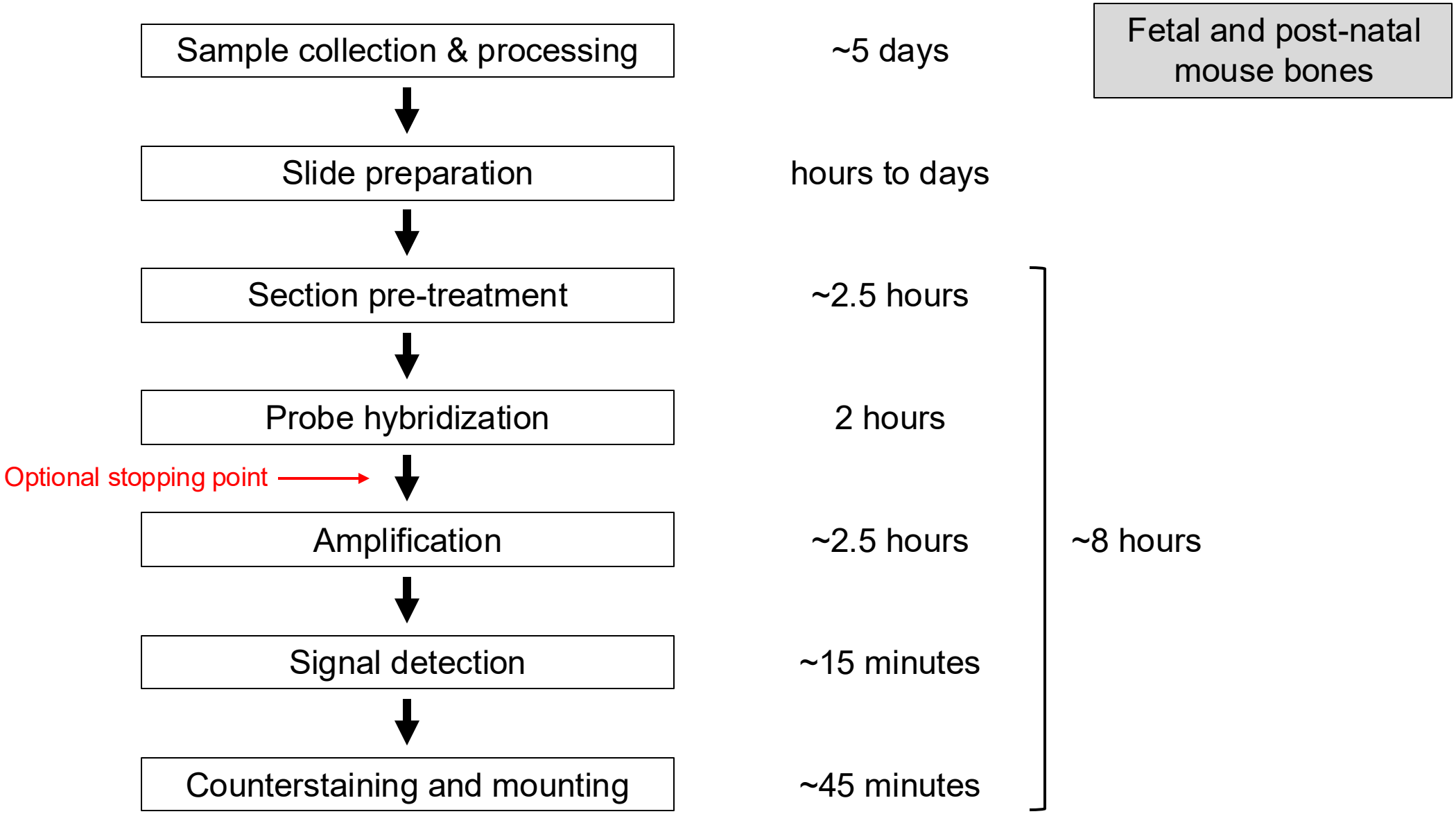
- All the assays can be performed **manually** or by automated systems
- For multiplex assays probes for different targets have to be designed with different channels

Reagents for RNAscope 2.5HD Red Assay protocol

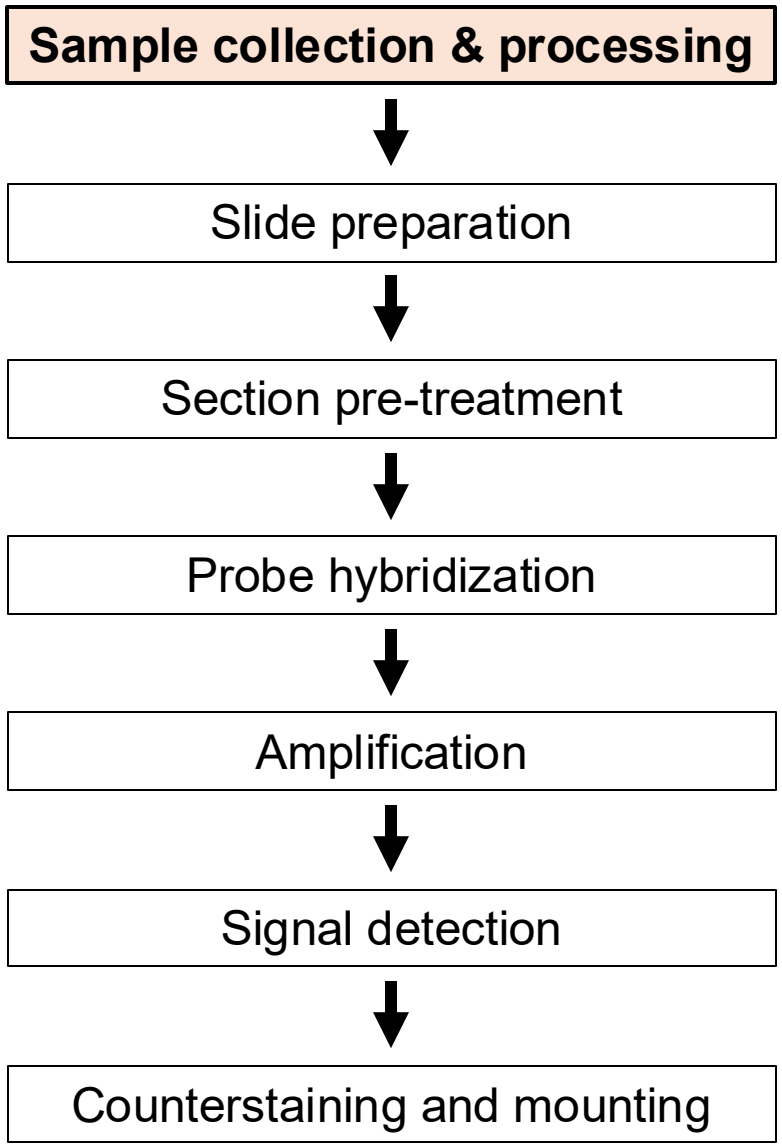
- **Equipment**
  - Dissection tools
  - Tissue processor and embedding station
  - Microtome
  - Water bath
  - Slide glass and coverslips
  - Drying oven
  - **HybEZ oven and tray**
  - **HybEZ slide rack (20 slides)**
  - Microscope

- **Reagents**
  - Paraformaldehyde
  - Sodium citrate and formic acid
  - Xylene and ethanol
  - Paraffin
  - Dietyhyl pyrocarbonate
  - Water and PBS (RNase free)
  - Hydrogen peroxide
  - **Custom Pretreatment reagent**
  - **RNAscope 2.5HD Red Assay kit**
  - **RNAscope Wash buffer**
  - **Probes for target RNAs (channel 1)**
  - Gill's Hematoxylin I
  - Ammonium hydroxyde
  - Permanent, polymer-based mounting medium

RNAscope 2.5HD Red Assay protocol for FFPE samples



Sample collection and processing



Fixation and demineralization

- Samples are fixed in **4% paraformaldehyde (PFA)** in phosphate-buffered saline (PBS) for 2 days at room temperature.
  - **Fix the samples right away** after animal euthanasia. The more you wait the more the RNA degradation occurs…
  - If your fixing long bones, make the bone marrow space accessible to the solution (e.g., make a cut at one epiphysis).
- Demineralization is performed in **Morse's solution** (24% formic acid in 10% sodium citrate) for 1 day at room temperature.
- All solutions should be prepared with diethyl pyrocarbonate (DEPC)-treated water.
- Fixation and demineralization times may have to be adjusted to the type of sample (e.g., embryo vs adult, mouse vs rat).
  - Signals for most RNAs were still detectable after 2 days of Morse's.

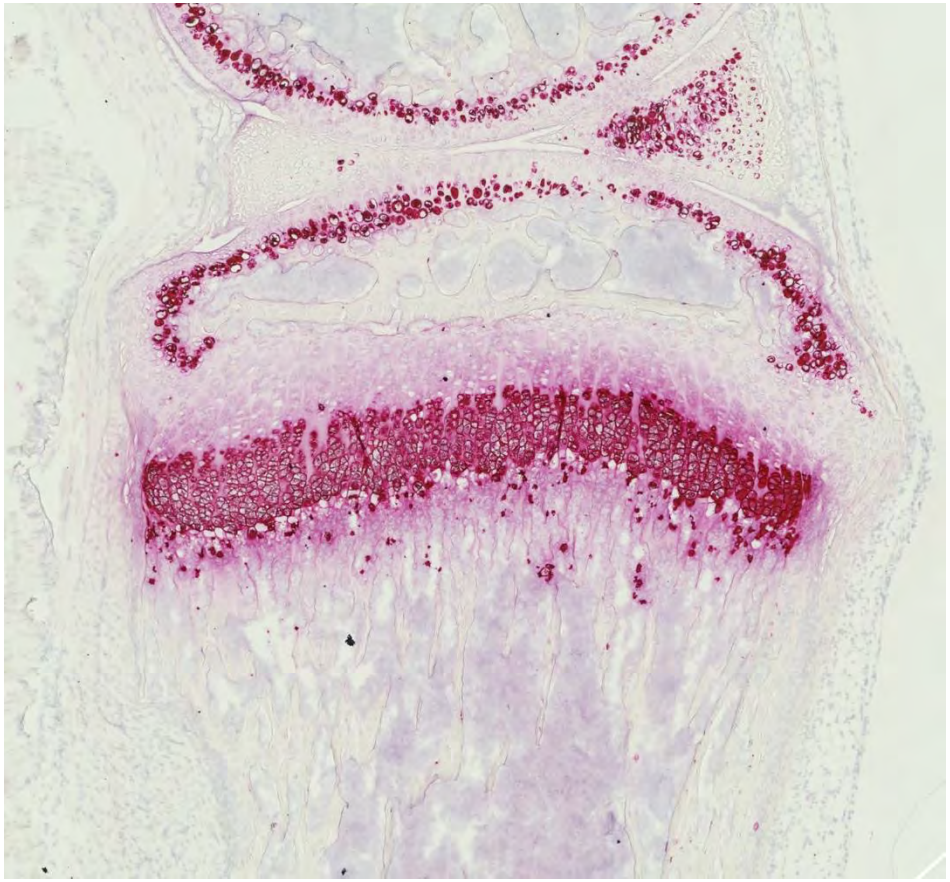
Why Morse's instead of EDTA?

- Morse's solution prevents RNA degradation better than EDTA
- Faster processing: 1 day (Morse's) vs 7-10 days (EDTA)

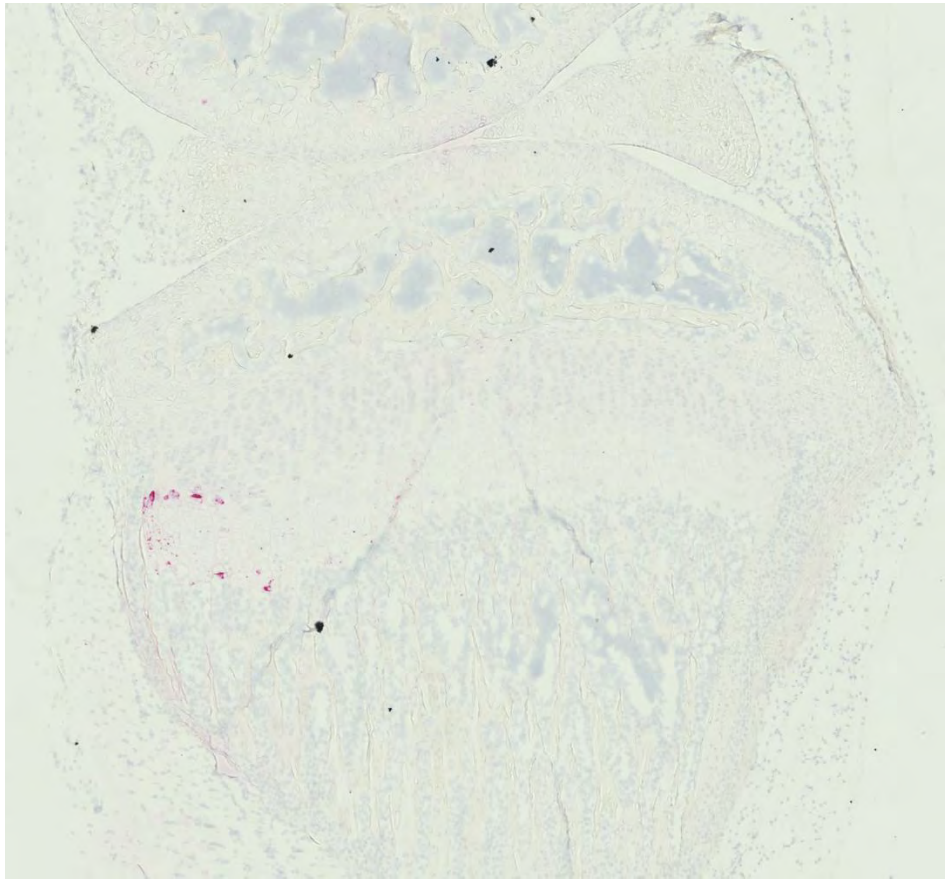
P22 mouse tibia

Col10a1

Morse's solution - 1 day



15% EDTA - 7 days

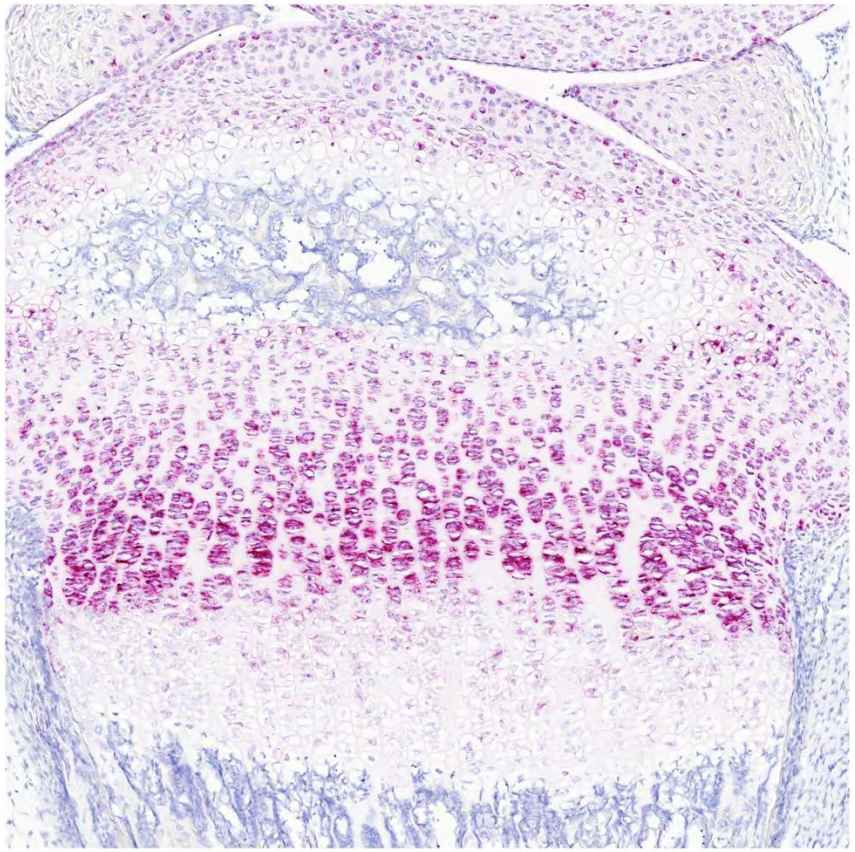


Our processing is compatible with immunostaining assays

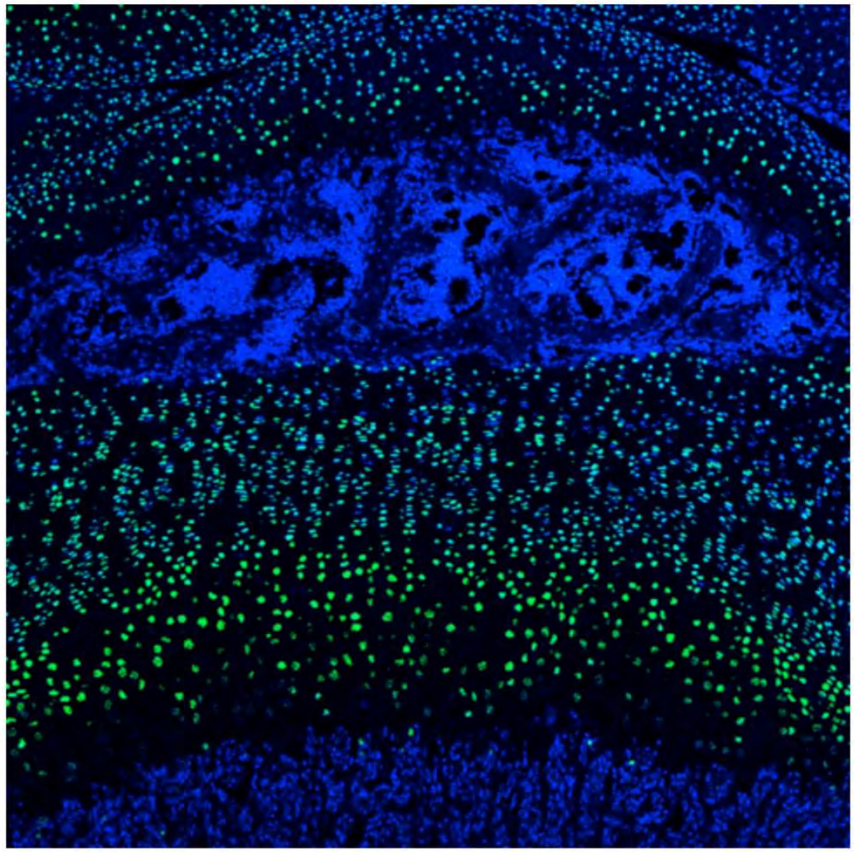
- Use the same samples for RNA and protein detection assays
- Multiplexing RNA and protein detection on the same slides

P15 mouse tibia

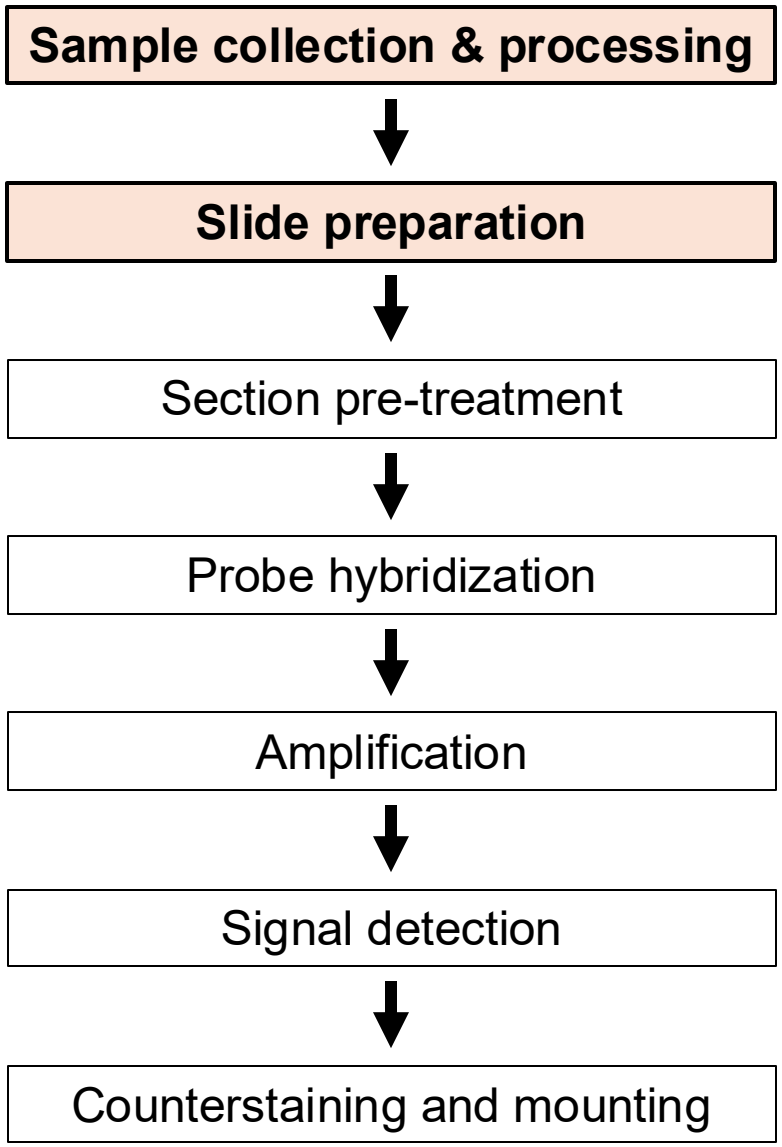
***Sox9* mRNA**



SOX9 protein



Sample collection and processing



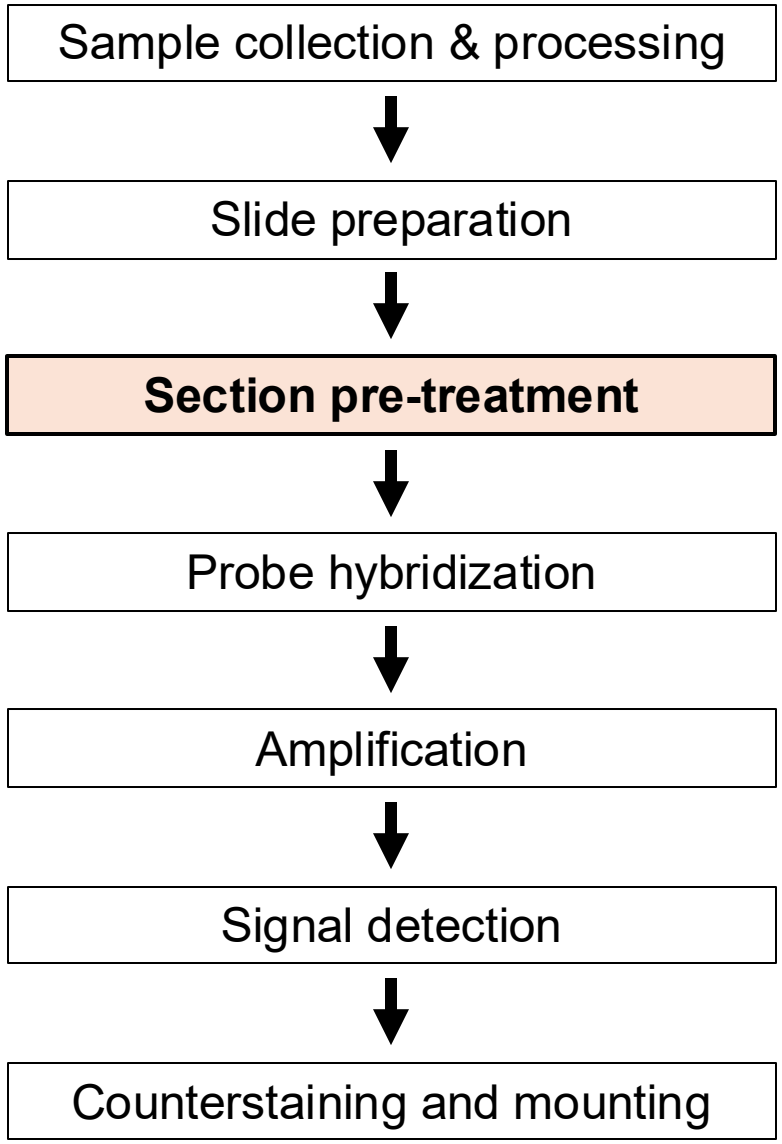
Paraffin embedding and sectioning

- Dehydrate, clear and embed in paraffin your samples.

Solution	Time
70% EtOH	1 h
70% EtOH	1 h
70% EtOH	1 h
95% EtOH	1 h
100% EtOH #1	2 h
100% EtOH #2	2 h
Xylene #1	1.5 h
Xylene #2	1.5 h
Paraffin #1	2 h
Paraffin #2	2 h

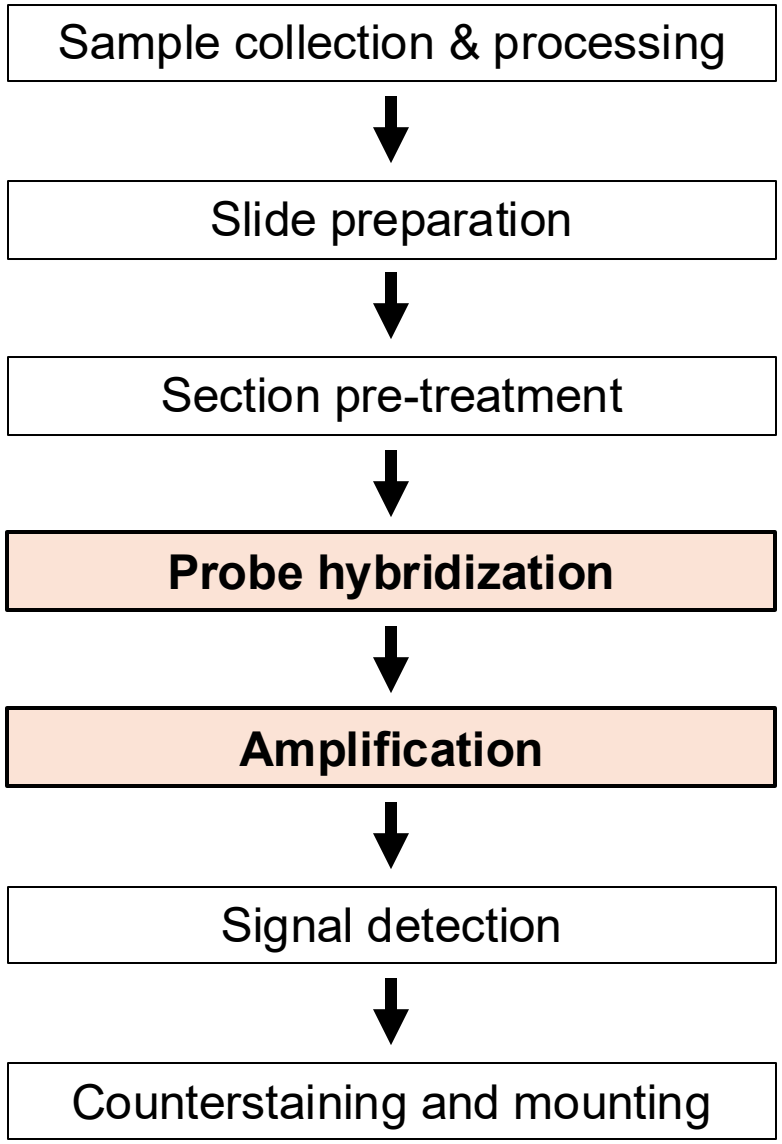
- Prepare 5-10 µm sections (42 ºC water bath) and dry them for 1 h at 37 ºC followed by overnight at room temperature.
  - Blocks and sections can be stored at room temperature.
  - Too old sections may not be suitable for RNAscope assays.

Section pre-treatment



- Bake the slides for 1 h at 60 ºC before deparaffinization and rehydration.
  - Baking the slides helps avoid section folding and detachment.
  - For deparaffinization and rehydration perform 2 incubation in xylene and 2 incubation in EtOH 100% (5 min each).
- Block endogenous peroxidases with 0.3% H_2O_2 (10 min at RT).
  - Use ACD Hydrogen Peroxide Reagent or make your own solution.
- Perform target retrieval using **Custom Pretreatment Reagent** (#300040) (30 min at 40 ºC in the HybEZ Humidity Control Tray).
  - The original protocol uses Target Retrieval and Protease Plus Reagent in a steamer. **Too harsh for sections from skeletal tissues!!! Sections tend to fold or detach.**
  - Retrieval with Custom Pretreatment Reagent may not be as efficient for low expressed genes.

Probe hybridization and amplification



- Probes and amplification reagents have to be equilibrated before use:
  - Probes have to be warmed at 40 ºC for at least 10 min.
  - Amplification reagents have to be placed at RT.
- Probe hybridization is performed in the HybEZ Humidity Control Tray at 40 ºC for 2 h.
  - After probe hybridization, slides can be stored in 5X SSC Buffer overnight (optional stopping point).
- Amplification is performed in 6 steps (AMP1 to AMP6). The first 4 are performed at 40 ºC, the last 2 at RT.
  - **AMP5 incubation time can be extended to obtain stronger signals.** Be aware that this could also increase the background noise!!!

Signal detection

Sample collection & processing

↓

Slide preparation

↓

Section pre-treatment

↓

Probe hybridization

↓

Amplification

↓

Signal detection

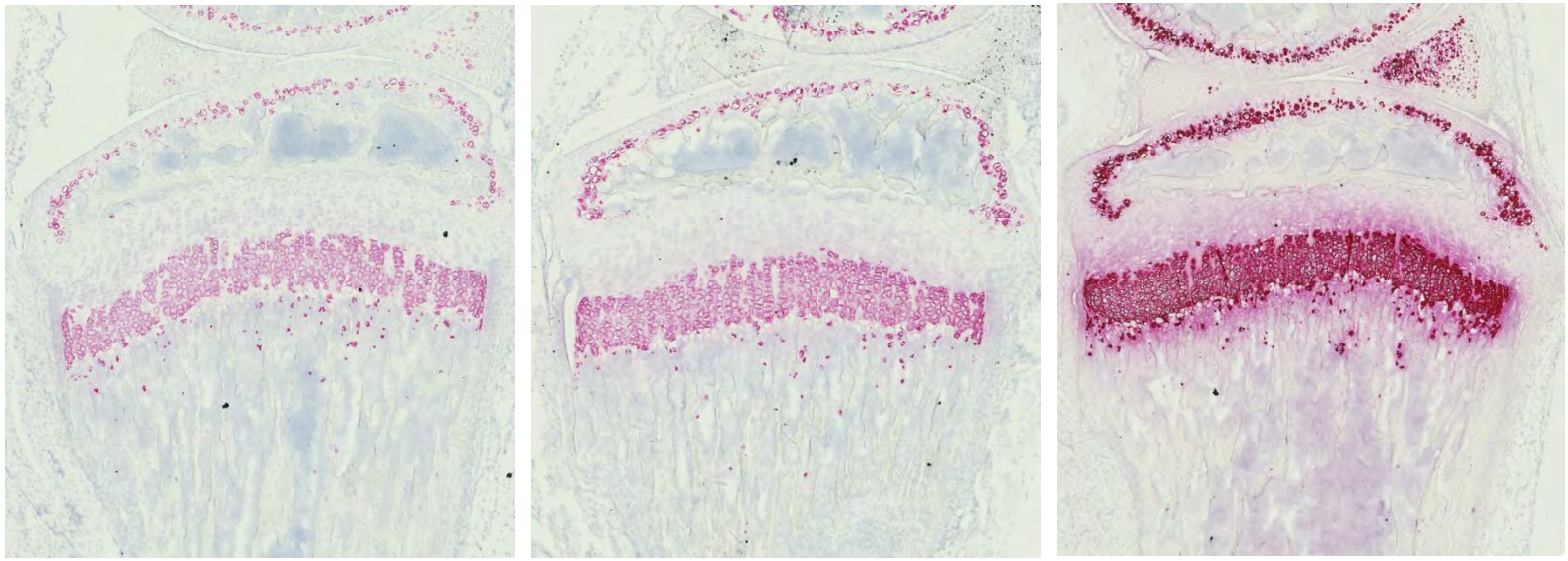
↓

Counterstaining and mounting

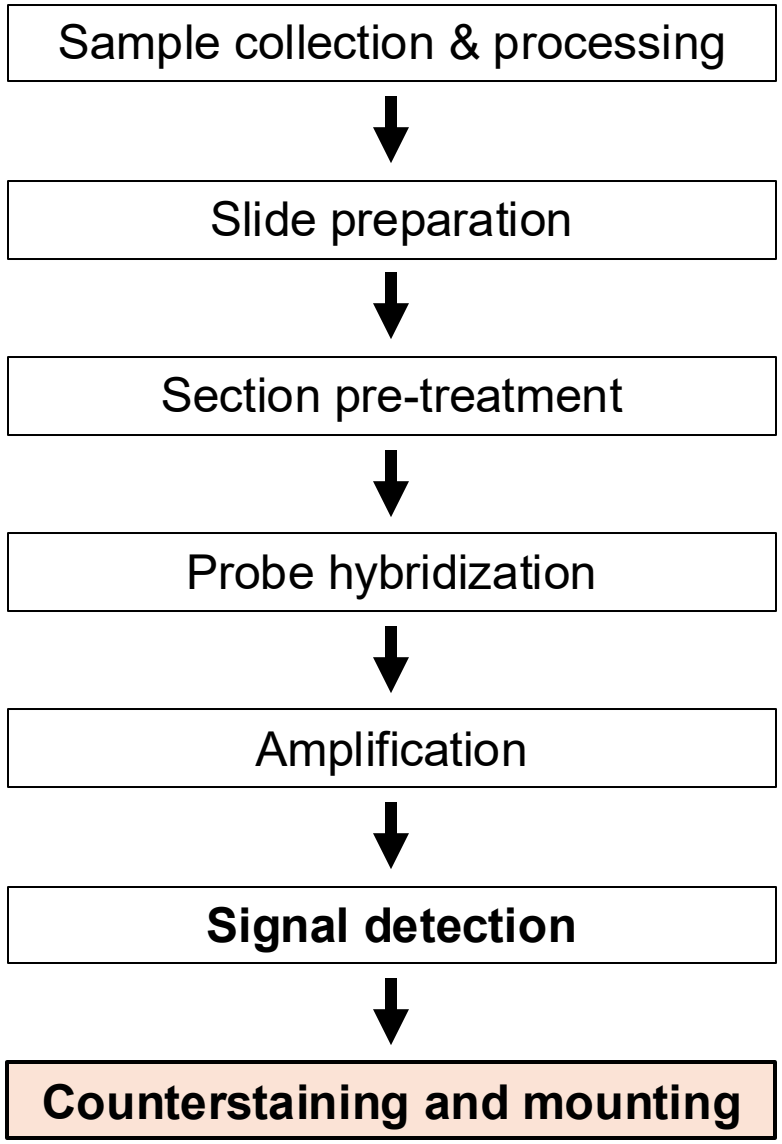
- Mix the Fast RED-A and RED-B reagents (ratio 1:60) and apply on sections within 5 min.
- Incubate for 10 min at RT.
  - **Incubation times for each probe can be changed to obtain weaker or stronger signals.** Be aware of the background noise when extending the incubation time!!

Col10a1

45 s | 90 s | 10 min



Counterstaining and mounting



- Counterstain slides with 20% Gill's Hematoxylin I and then perform bluing in 0.02% ammonia water.
  - The original protocol suggests a 50% hematoxylin solution. We use a 20% to obtain a **lighter counterstain to avoid hiding weaker red signals**.
- Dry slides at 60 ºC in an oven for 15-30 min and mount with permanent, polymer-based mounting medium after quick dip in xylene.
  - **Do not dehydrate the slides in ethanol, since the RED substrate is alcohol sensitive.** The BROWN substrate is instead compatible with ethanol.
- Once the mounting medium is dry, visualize the slides and take pictures.
  - **The red dye can also be visualized as a fluorescent signal.** In that case counterstain with DAPI.

Protocol published in Methods Mol Biol. 2021 as chapter 6 of the "Chondrocytes" book

Preparation of Adult Mouse Skeletal Tissue Sections for RNA In Situ Hybridization

Charles de Charleroy, Abdul Haseeb, and Véronique Lefebvre

Thanks for your attention

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