Protocol: Combination RNAscope/GeoMx on Bone w/ Brown DAB Kit

Washington University Musculoskeletal Research Center (MRC)
Last Updated: 9-29-23
Contributors: Silva Lab (Chris Chermside-Scabbo) and Scheller Lab (Alec Beeve, Katherine Minielly), with additional tips and input provided by Dr. Thomas Andersen (University of Southern Denmark)

This protocol can be used for single gene RNAscope and/or GeoMx spatial transcriptomics. Similar principles apply for RNA probe binding with other spatial techniques (e.g. 10x Visium, CosMx, etc). Prior to transitioning to spatial techniques, we recommend optimizing individual gene RNAscope using sections from your exact samples/blocks of interest. If individual control and/or target genes are staining well and in expected patterns, your transition to multi-probe binding using serial sections from the same blocks (e.g. 21,000 probes in the GeoMx whole transcriptome atlas) is highly likely to succeed. If stains for single genes are not working well, we recommend additional optimization or fresh sample collection and testing prior to moving forward.

Video Resources:

- Individual videos: https://acdbio.com/technical-support/learn-more/training-videos
- Overview: https://www.youtube.com/watch?v=EUSgkbAgdHA
- How to Use EZ-batch tray: https://www.youtube.com/watch?v=7NZ0rtVwgDw

Processing Date	Samples Processed		Controls
			Pos Control (UBC or PPIB): Neg Control (DapB):

RNAscope Material Checklist (for up to 20 slides):

1. Coated glass slides (Agilent Dako, IHC FLEX, K8020) – used to promote section retention
2. ACD RNAscope 50X Wash Buffer [1 entire bottle] (ACD #310091)
3. ImmEdge Pen (Vector Labs #H-4000)
4. VectaMount
5. RNAscope H_2O_2 (ACD #322335)
6. Pepsin (Sigma #R2283)
7. Ammonium hydroxide (Sigma #338818-5mL)
8. Gill's Hematoxylin (Sigma #GHS132-1L)
9. RNAscope 2.5 HD Detection Reagent (ACD #322310)
10. P200 & P20 w/ tips
11. Autoclaved & dried histology staining containers [can dry overnight in oven] (8)
12. Autoclaved large (3L) container
13. Xylenes [at least 800mL]
14. 100% EtOH [at least 800mL]
15. 95% EtOH [at least 800mL]
16. PBS [at least 500mL]
17. NBF Stop Buffer: Add 24.5 g Tris base and 15 g Glycine to 2L of milliQ water
18. Cover slips (Fisherbrand #22266858)
19. Grace Bio-Labs HybriSlip (sized to cover your sample)

Tissue Collection and Processing

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Note: Rapid fixation is required for optimal preservation of target antigens. Example protocol provided below – this worked for mouse tibiae.

1. Euthanasia method: CO2 + cervical dislocation
2. RNAse free conditions: use RNase away on the bench and spray the tools
3. Mouse perfusion at dissection? None
    a. Note: Perfusion with formalin may promote antigen preservation
4. Tissue isolation: Bilateral tibiae were dissected and trimmed to remove excess muscle without disrupting the periosteum
5. Tissue fixation solution: 10% neutral buffered formalin
6. Tissue fixation time: overnight 24-hours at room temperature, put on a rocker shaker (gentle agitation)
7. Tissue fixation volume: 1 bone per 15 mL conical filled to the top with formalin
    - Tissue washing: wash in PBS three times for ~20-30 minutes each with gentle agitation, keep bones in the same tube
8. Decal: keep bones in the same tube, add 14% EDTA, pH 7.2 (fresh, made within 2-weeks), change it every 2- to 3-days, total decal time is 10 to 14 days
    - Note: Adding 10% formalin to your EDTA during decal at 1:10 ratio may help to promote RNA antigen preservation (tip from the Andersen Lab, Denmark)
9. Tissue washing: keep bones in the same tube, wash in milliQ water three times for 20-30 minutes with gentle agitation on a rocker shaker
10. Tissue dehydration: use a graded ethanol series 30%, 50%, 70% with 3 washes each for 20-30 minutes with gentle agitation
11. Submit in 70% ethanol for paraffin embedding, maintain RNase free processing as is possible
12. Cut 3 to 5 um sections onto coated glass slides (Agilent Dako, IHC FLEX, K8020)
    - Note: we found that the coating was essential to promote optimal bone section retention. This is not necessary for other tissue types.
13. Use cut slides for RNAscope or spatial transcriptomics within 2-weeks

Prep At Some Point [Can easily be day of during 15min oven steps]

Prepare 1X Wash Buffer

1. Warm 50X wash buffer for 10min in 37-40°C water bath [in tissue culture room]
2. Dilute in 3L pyrex bottle
    - Dispense 2940mL of milliQ water into bottle
    - Dump in 50X wash buffer (60mL) & stir by inverting
3. Store @RT for up to 1 month

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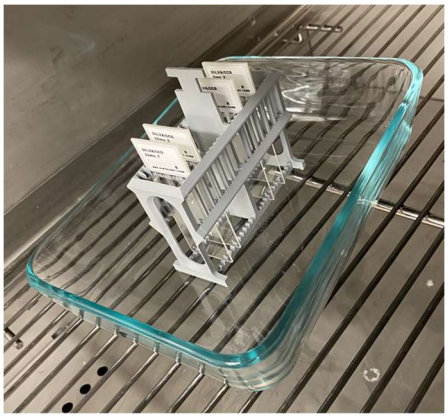


Prep Day Before

RNAscope Slides:

1. Ensure slides have dried overnight in plastic bag + desiccant
2. *Night before starting*: Bake slides at 60°C overnight
    - Important to promote section retention
    - Vertically in slide holder

Day of:

Setup (Bench Top & Oven): _____ **Time:** _____

1. Wash bench top, fume hood, and pipettes with ETOH and RNase Away ▢
2. Place Pretreat #1 (H_2O_2) and pepsin @RT ▢
3. Prepare HybEZ Oven wet chamber.
    - Turn ON (switch is on the back of the machine).
    - Should auto-set to RNAscope 40C. Change mode if not.
    - Wet humidifying paper with DI or milliQ water and enclose in humidifying tray. Place into oven to pre-heat.
4. Place vertical glass slide holders (make sure you put enough holders to hold each slide you're processing) into the water bath near the HybEZ oven. Make enough fresh 1x Target Retrieval Reagent to fill the glass slide holders (each holds ~50mL). Fill slide holders with Target Retrieval Reagent and turn on the water bath. Make sure it reaches 75-78C.
5. Place 500-1000mL bottle of milliQ water into 60C bead bath in cell culture room.

Deparaffinization & Dehydration + Pretreatment #1 [1.5hr]

6. Prepare containers with fresh Xylene (3), 100% EtOH (2), 95% EtOH (2), and PBS (1) ▢
7. Deparaffinize slides in fresh Xylene
    - 3 containers 5min each ▢▢
8. Rehydrate slides in fresh 100% EtOH
    - 2 containers 5min each ▢▢
9. Rehydrate slides in fresh 95% EtOH
    - 2 containers 5min each ▢▢
10. Wash slides in fresh PBS.
    - 1 containers 1min ▢

- Can store in PBS for up to 1 hour.

11. Pretreatment #1 [hydrogen peroxide]: drop H202 from ACD (or 1.5% hydrogen peroxide in ethanol) to cover your samples on the slides.
    - Incubate for 15 minutes at RT ▢
12. Dip in PBS (from the 2nd container, step #10 above) ▢
13. Dry slides on a Kimtech wipe at RT for 5min ▢
    - Remove slides from slide rack and place tissue side up

*Pretreatment 2 [Kit Reagent] [15min]*_____**Time: _____**

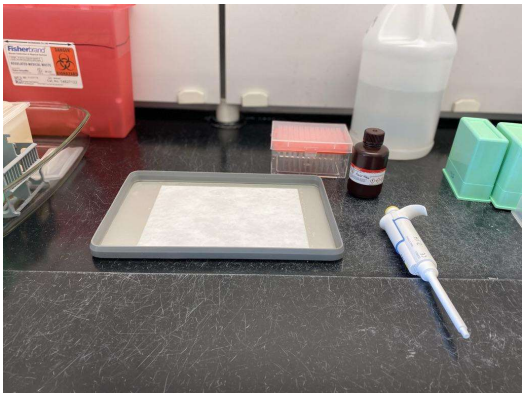
Note: Heat antigen retrieval below is optional. We get good results in the periosteum with only pepsin antigen retrieval. However, in our hands, heat pretreat #2 is beneficial and helps with antigen retrieval for osteocytes and especially in bone marrow.

14. Submerge slides in MilliQ water pre-heated to 60°C for about 10 seconds.
15. Incubate slides in pretreatment #2 for 20 minutes at 75-78°C (monitor temp)
16. Submerge in MilliQ water pre-heated to 60°C 2 x 3 minutes
    - 2 containers 3min each ▢▢
17. Place slides into EZ-batch tray ▢

Pretreatment 2 [Pepsin] [50min] **Time: _____**

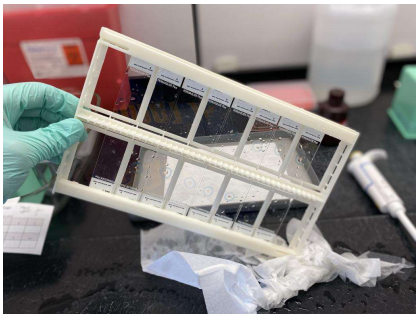
IN FUME HOOD:

18. Prepare P200, tips, and hybridization chamber in hood



19. Dry off slides as much as possible by tapping against kimtech wipes on counter
20. Cover slides in pepsin using P200
21. Cover chamber and incubate for **20-45** minutes **@40°C** in HybEZ oven ▢
    - Note: This should be *optimized* on a per sample basis. If performing pre-treat #2, can go as high as 30 minutes, with 20-minutes found to be optimal in prior trials. Without pre-treat #2, can go as high as 45 minutes.
        i. Best outcome: good section retention with strong RNAscope stain
        ii. Poor outcome (under digestion): poor RNAscope stain

iii. Poor outcome (over digestion): fragmentation and tissue loss



22. With ~10min remaining, warm probe(s) in **37-40°C** bath [in tissue culture room] ▢

23. Wash slides in milliQ H_2O at room temp by decanting pepsin and submerging for 3min x2 washes @RT ▢▢
    - This can be done directly in the slide holder tray
24. Dry off slides as much as possible by tapping against kimtech wipes on counter
25. *Optional: Place slides back in oven for 30min @60°C [can help with tissue adherence]*
    - *Often do this for 1-2 min with the whole tray while I get the probes ready*

*Postfix [20 minutes]*_____**Time: _____**

26. Prepare containers with fresh 10% NBF (1), NBF Stop Buffer (2), and PBS (1) ▢
    - NBF Stop Buffer: Add 24.5 g Tris base and 15 g Glycine to 2L of milliQ water
27. Postfix slides in fresh 10% NBF
    - 1 container 5min ▢▢
28. Stop fix in fresh NBF Stop Buffer
    - 2 containers 5min each ▢▢
29. Wash slides in fresh PBS.
    - 1 containers 5min each ▢▢
    - Can store in PBS for up to 1 hour.

*Hybridization [2hrs to overnight]*_____**Time: _____**

30. Drop 1-2 probes onto tissue ▢
    - Undiluted can work well for bone
    - Probes for bone can often also be diluted 1:2 (ex. 3 drops probe + 3 drops diluent) – this will allow you to run 2x the number of slides
    - Cover completely. Number of drops varies on size of sample.

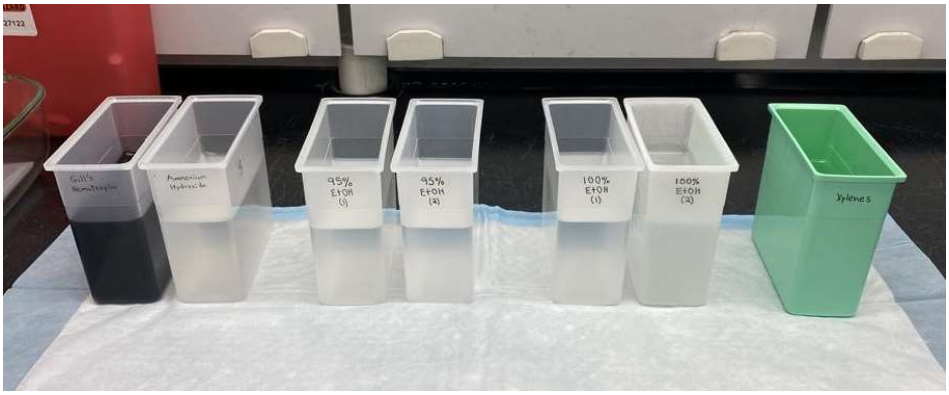
31. Apply Grace Bio-Labs HybriSlip gently by setting one edge of the coverslip down in solution on the slide, then gradually laying down the rest of the coverslip to avoid air bubbles.
28. Incubate in HybEZ oven for **2hrs @40°C (undiluted) or overnight @40°C (1:2 diluted)** ▢
    - Overnight incubations may also help undiluted probes to achieve optimal binding in your tissues
    - Overnight incubation is recommended for GeoMx spatial transcriptomics probes
    - Thicker tissues will require longer incubation times
    - These times are based on a tissue thickness of 3-5 µm

Continue to amplification and visualization for RNAscope probes below, or transition to GeoMx spatial transcriptomics recommended counterstains and processing (see manufacturer instructions).

Amplification [3hrs] **Time: _____**

29. Remove the HybriSlip from the slide. DO NOT force it off. Place the slide into fresh PBS and it should slide off. Improper post-fix will cause tissue distortion with this step.
30. After using AMP reagent, return it to bar cooler and acquire the next to equilibrate
    *Protocol says you can place all at RT simultaneously *
31. Wash slides in fresh 1X Wash Buffer using EZ-batch tray for 2min @RT (x3) ▢▢▢
32. Apply AMP1 onto all slides & Incubate in HybEZ oven for **30min @40°C** ▢
33. Wash slides in fresh 1X Wash Buffer using EZ-batch tray for 2min @RT (x2) ▢▢
34. Apply AMP2 onto all slides & Incubate in HybEZ oven for **15min @40°C** ▢
35. Wash slides in fresh 1X Wash Buffer using EZ-batch tray for 2min @RT (x2) ▢▢
36. Apply AMP3 onto all slides & Incubate in HybEZ oven for **30min @40°C** ▢
37. Wash slides in fresh 1X Wash Buffer using EZ-batch tray for 2min @RT (x2) ▢▢
38. Apply AMP4 onto all slides & Incubate in HybEZ oven for **15min @40°C** ▢
39. Wash slides in fresh 1X Wash Buffer using EZ-batch tray for 2min @RT (x2) ▢▢
40. Apply AMP5 onto all slides & Incubate on bench top for 30-**60min @RT** ▢
    - Extending this to 60 minutes can increase the signal without increasing the background
41. Wash slides in fresh 1X Wash Buffer using EZ-batch tray for 2min @RT (x2) ▢▢
42. Apply AMP6 onto all slides & Incubate on bench top for **15min @RT** ▢
    - Prepare the DAB mixture [see below] ▢
    - Prepare the Gill's hematoxylin & Ammonium Hydroxide water [see below] ▢

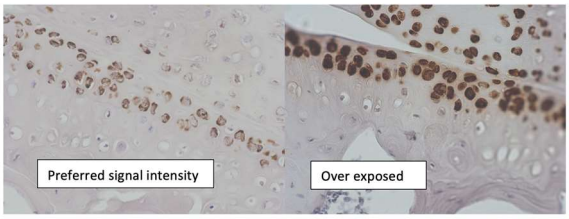
- Prepare Ethanols and Xylenes [see below] ▢



43. Wash slides in fresh 1X Wash Buffer using EZ-batch tray for **2min @RT (x2)** ▢▢

Option #1: DAB Colorimetric Reaction [15-20min] **Time: _____**

44. Prepare the DAB mixture by mixing DAB-A & DAB-B 1:1 ▢
    - Add an equal number of drops to a 1.5mL eppendorf (1 * n slides of each A & B)
45. Apply DAB by pipetting to sample individually for ~10min ▢
    - Reaction can be very fast and should be monitored when using new probe
46. Stop the DAB reaction by immersing the slides into fresh Tap water (yes, Tap!) ▢
    - Use EZ batch tray to quence all simultaneously
    - Shake the slide slightly to wash off DAB



Option #2: TSA + DIG (~5000x increase in sensitivity, requires an additional kit) [1.5-2.0 hours]
Time: _____

47. This method uses horseradish peroxidase (HRP, already present after the amplification steps) to catalyze the covalent deposition of digoxigenin (DIG) labels directly adjacent to the immobilized enzyme. It will significantly amplify your signal and can be useful for low-abundance transcripts. You will need to purchase additional staining reagents in addition to the standard RNAscope Brown DAB kit. Specifically:
    - TSA Plus DIG – Akoya Biosciences (NEL748001KT)
        i. https://resources.perkinelmer.com/lab-solutions/resources/docs/FLY_TSA-Order-Guide.pdf?_ga=2.159622904.450302829.1669734691-103369431.1669734691

ii. https://www.fishersci.com/shop/products/akoya-tsa-plus-dig-50-150slide/NC1963598#?keyword=TSA%20PLUS%20DIG,%2050-150%20SLIDES

- Anti-Digoxigenin-AP, FAB fragment from Roche (11 093 274 910)
  i. https://www.sigmaaldrich.com/US/en/product/roche/11093274910
  ii. https://www.fishersci.com/shop/products/anti-digoxigenin-ap-co-200-ug/501003276#?keyword=Anti-Digoxigenin-AP
- Vector Laboratories ImmPACT™ VECTOR Red Alkaline Phosphatase (AP) Substrate Kit (SK5105)
  i. https://www.fishersci.com/shop/products/red-substrate-120ml-alk-phos/NC0771001#Liquid%20permanent%20red%20chromogen
  ii.

48. Wash slides in TBS/TWEEN using EZ-batch tray for **2min @RT (x2)** ▢▢
49. Add TSA plus DIG reagent (1:250 in 1x plus amp diluent) **5min @RT** ▢
50. Wash slides in TBS/TWEEN using EZ-batch tray for **5min @RT (x2)** ▢▢
51. Add Anti-DIG-AP 1:1000 (no.214) for **60min @RT** ▢
52. Wash in fresh Tap water (yes, Tap!) **2min @RT (x2)** ▢▢
53. Add liquid permanent red to develop stain, freshly prepared **10min @RT** ▢
    - Reaction times should be monitored when using a new probe to achieve optimal staining
54. Wash in fresh Tap water (yes, Tap!) to stop reaction **5min @RT (x1)** ▢

Counterstaining [5min] **Time: _____**

55. In fume hood, prepare [above] Gill's hematoxylin by:
    - Dispensing 100mL of milliQ H_2O into slide staining container ▢
    - Adding 100mL of Gill's hematoxylin using serological pipette w/ 25mL pipette ▢
      * Can be reused for up to 1 week *
56. In fume hood, prepare [above] Ammonium Hydroxide water by:
    - Dispensing 200mL of milliQ H_2O into slide staining container ▢
    - Pipetting 143uL of 28% Ammonium Hydroxide into slide container and mix ▢
57. Move slides to slide holder filled with tap water
58. Counterstain with hematoxylin for 1-2min (or less, hematoxylin is quite strong) ▢
59. Wash in tap water
60. Place sections in Ammonia Water until sections turn blue (8 [3-10] dips) ▢
61. Wash in tap water ▢

Dehydration [10min] **Time: _____**

62. Prepare Ethanol & Xylenes:
    - Obtain 5 staining containers
    - 95%: In 500mL beaker, add 20mL milliQ H20 & top off to 400mL w/ 100% Ethanol
      i. Mix & distribute to 2 staining containers
    - 100%: Fill 2 staining containers with 100% Ethanol

- Xylenes: Fill 1 staining container with Xylenes

63. Dehydrate in graded Ethanols
    - 95% for 2min x 2 ▢▢
    - 100% for 2min x 2 ▢▢
64. Clear slides in fresh Xylene for 5min ▢

Mounting [10min] **Time: _____**

65. Obtain cover slips & set in easy-to-grab spot
66. One at a time, remove slides from xylene
67. Without the Xylenes drying, immediately pipette 10uL of VectaMount
68. Place coverslip
69. Wick away extra Xylenes on paper towel & use forceps pressing down to remove bubbles
70. Allow to dry for >5min

Imaging

71. Power on Confocal using brightfield mode
72. Alternatively, image on Nanozoomer (recommended)