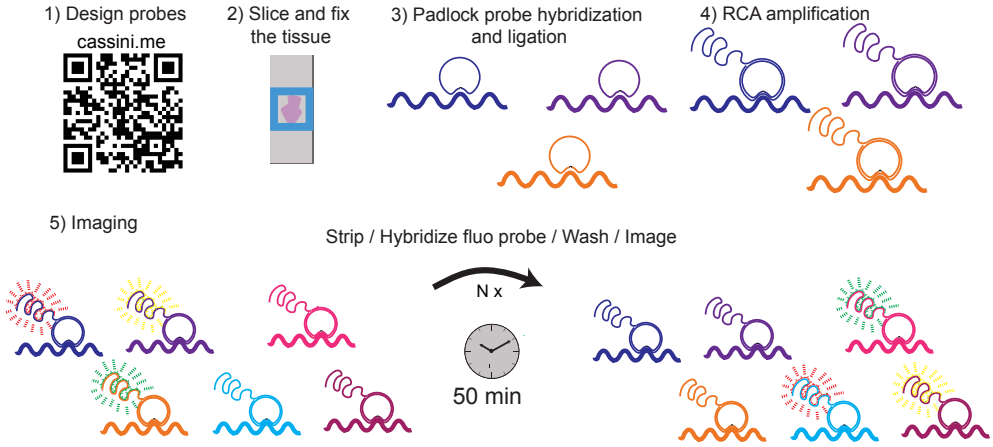
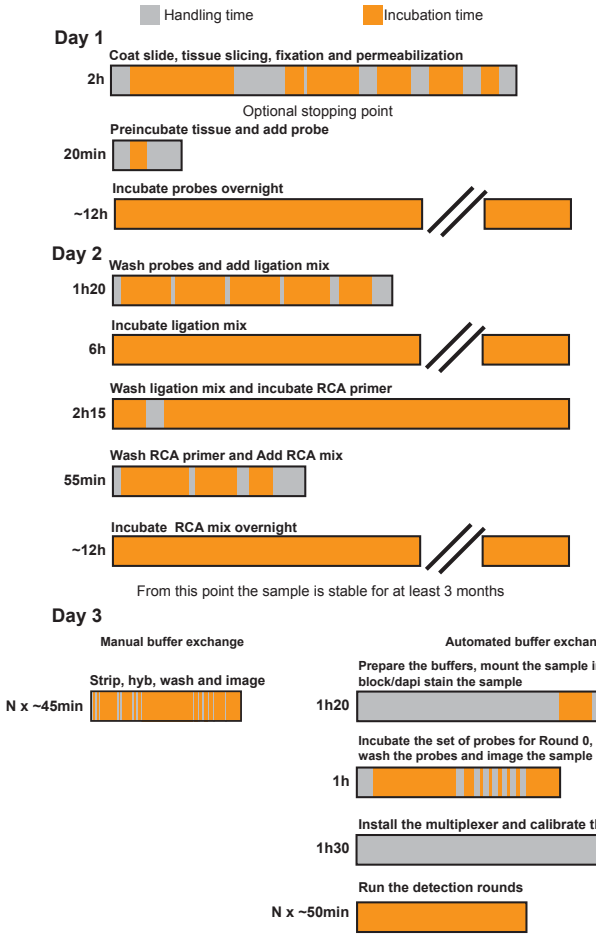
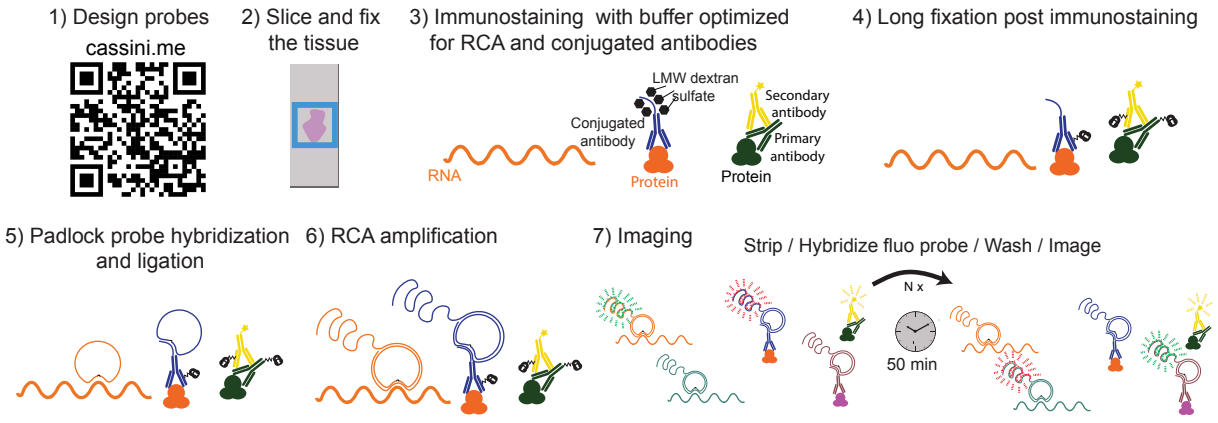
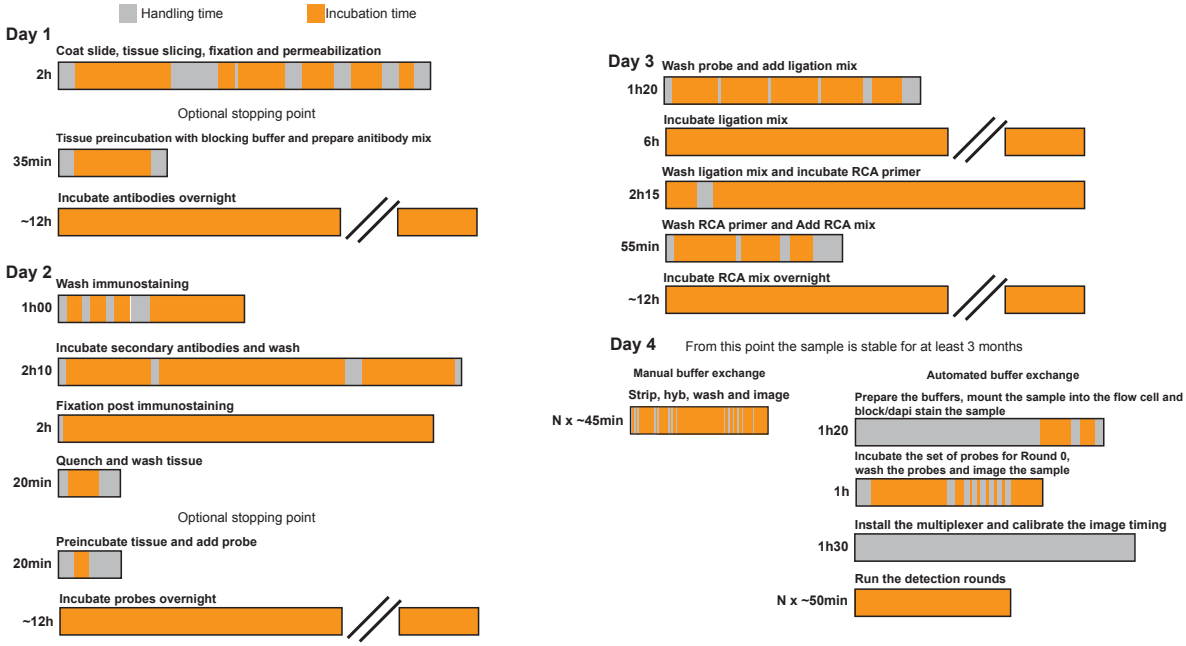
Cassini workflow for RNA only





Cassini workflow for multimodal RNA, conjugated and regular immunostaining





Common reagents for RNA and immunostaining

- Padlock probes : **Padlock probes need to be phosphorylated on the 5'**
- Fluorescent probes
- RCA primer- **RCA primer needs 2 phosphorothioate bonds on the 3' to prevent degradation – e.g. CCTGTGTGAGTCTCC*T*G**
- PBS
- Ultra pure Water
- Tris-HCL pH7.5 1M
- HCl 1M
- Poly-L-Lysine Solution (0.01%) (Sigma A-005-C)
- 40mm glass coverslips (Bioptechs Inc)
- 9x9mm hybridization chamber (Biorad SLF0201)
- 4% PFA (Thermo Fisher Scientific J61899.AK)
- Triton X (Sigma X100-5ML)
- RNAse inhibitor (Y9240L Qiagen)
- Formamide (Sigma Aldrich F7503-4L)
- 20XSSC (Thomas Scientific C000A15)
- Dapi (Thermo Fisher 62248)
- High molecular weight dextran sulfate (Sigma Aldrich S4031)
- Ultra pure BSA 5% (Invitrogen 56773)
- SplintR Ligase (NEB M0375L)
- Phi29 (Thermo Fisher EP0094)
- dNTPs (NEB N0447L)

Reagents for immunostaining (regular + conjugated)

- FcR Blocking Reagent (Mouse: Miltenyi biotec #130-092-575, Human:Miltenyi biotec #130-059-901)
- Ultra pure BSA 5% (Invitrogen #56773)

Reagents for conjugated immunostaining

- Dextran sulfate-4K (Sigma #75027)
- NaCl 1M
- oYo-Link® Oligo Custom (AlphaThera)

Reagents for regular immunostaining

- Normal serum specific to the species of the secondary
- Fluorescent secondary antibody

Hardware Material

Sample preparation (Optional)

- HybEZ™ II Oven (ACDBio)

Automated buffer exchange (Optional)

- FCS2 flow cell (Bioptechs)
- 2 Rotary valves (AMF)
- Raspberry pi
- Peristaltic pump (Kamoer KCM-ODM-B253)

Tissue preparation

Material

- Ultra pure Water
- Poly-L-Lysine Solution (0.01%) (Sigma A-005-C)
- 40mm glass coverslips (Bioptechs Inc)
- 9x9mm hybridization chamber (Biorad SLF0201)

Protocol

1. Coat the round glass with a solution of Poly-L-Lysine Solution (0.01%) for 30 minutes. Wash them by immersion in Ultra pure H2O and let them dry until all moisture has evaporated. If you want to speed up the drying process, you may use compressed air. During the incubation time, place the fresh frozen brain in the cryostat at -18˚C to equilibrate (at least 20 minutes).

2. Add a hybridization chamber on each slides (Biorad) and press firmly with a plastic tool
   Critical step: The glass is very thin and can break easily if press on an uneven surface

3. Place the glass slides in the cryostat at -18˚C and wait for 5 minutes before slicing. Slice a 10um thick tissue brain section and melt it on the glass slide with the help of a finger. Let the slide equilibrate at RT from 2 minutes in a humidified chamber before processing to the next step.

Tissue fixation

Material

- PBS
- Ultra pure Water
- 4% PFA (Thermo Fisher Scientific J61899.AK)
- Tris-HCL pH7.5 1M
- Triton X (Sigma X100-5ML)
- HCl 1M

Mixes

Tris-HCL pH7.5 100mM (Solution is stable at room temp for long term storage)

- 900 µl of ultra pure water
- 100 µl Tris-HCL pH 7.5 1M

Triton 0.25% (Solution is stable at room temp for long term storage)

- 10 ml of Ultra pure water
- 25 µl of Triton X → To facilitate easier pipetting of the Triton solution, we recommend trimming the ends of the pipette tips.

HCl 100mM (Solution is stable at room temp for long term storage)

- 900 µl of Ultra pure Water
- 100 µl of HCl 1M

Protocol

4. Add 50 µl of PFA 4% and incubate for 12-14 minutes at room temperature and wash 3 times with 80 µl of PBS

5. Add 50 µl of 20mM Tris-HCL to quench the PFA and incubate 10min at room temperature and wash 3 times with 80 µl of PBS

6. Add 30 µl of 0.25% Triton and incubate 10min at room temperature and wash 3 times with 80 µl of PBS

7. Add 50 µl of 0.1M HCl and incubate 5min at room temperature and wash 3 times with 80 µl of PBS

Immunostaining + 2nd fixation (Skip this step if RNA only)

Material

- PBS
- Ultra pure Water
- NaCl 1M
- Low molecular weight Dextran sulfate (Sigma Aldrich 75027)
- FcR Blocking Reagent mouse (Miltenyi biotec 130-092-575)
- Ultra pure BSA 5% (Invitrogen 56773)
- RNAse inhibitor (Y9240L Qiagen)
- Tris-HCl pH 7.5 100mM
- Antibodies (conjugated and/or conventional)

Mixes

20% Dextran sulfate-4K (Solution is stable at 4˚C for long term storage)

- 1g of Dextran sulfate-4K
- 5ml of ultra pure water

Blocking buffer 1 – Make it fresh

- 1 µl of FcR Blocking Reagent
- 5 µl of NaCl 1M
- 1 µl of RNAse inhibitor
- 10 µl of Ultra pure BSA 5%
- 5 µl of 20% Dextran sulfate-4K
- 78 µl of PBS

Blocking buffer 2 – Make it fresh

- 2.5 µl of FcR Blocking Reagent
- 12.5 µl of NaCl 1M
- 25 µl of Ultra pure BSA 5%

- 12.5 µl of 20% Dextran sulfate-4K
- 197.5 µl of PBS

PBS + RNAse inhibitor – Make it fresh
- 160 µl of PBS
- 1.6 µl of RNAse inhibitor

Secondary blocking buffer – Make it fresh (Only for conventional immunostaining)
- 5 µl of Normal serum specific to the species of the secondary
- 94 µl of PBS
- 1 µl of RNAse inhibitor

Protocol
All steps must be done in a humidified chamber !

8. Add 50 µl of blocking buffer 1 and incubate 30min at 4c.

9. Prepare a diluted solution of antibodies by incorporating them into the blocking buffer. To achieve optimal specificity, it is generally necessary to dilute the conjugated antibodies 100 to 200 more than standard antibodies. We typically dilute our conjugated antibodies to 100'000x. Remove previous solution and add 50 µl of the diluted antibodies to each sample and incubate overnight at 4C.

10. Wash 3 times with 80 µl of blocking buffer 2, with 5 min incubation at room temp for each wash.

11. Wash 3 times with 80 µl PBS with ~15 seconds of incubation for each wash and add 80 µl of PBS + RNAse inhibitor (0.4U/ µl) and incubate 30 min at room temp.

12. *Only for conventional immunostaining*: Incubate the sample with 50µl Secondary blocking buffer for 30min at room temperature. Dilute secondary antibodies in 50µl of secondary blocking buffer, remove the blocking solution, add the secondary mix to the sample and incubate 1h at room temp.

13. *Only for conventional immunostaining*: Wash the secondary antibodies 3 times with 80µl of PBS with ~15 seconds of incubation for each wash and incubate with 80µl PBS + RNAs inhibitor for 30 minutes at room temperature. Wash with 80ul of PBS.

14. Remove PBS and add 50 µl of PFA 4% and incubate for 2h at room temperature.

15. Remove the PFA and add 50 µl of 100mM Tris-HCL and incubate 10min at room temperature.

16. Wash 3 times with 80 µl of PBS, with 1 min incubation at room temp for each wash.

Probe hybridization

Material

- Ultra pure Water
- Formamide (Sigma Aldrich F7503-4L)
- 20X SSC (Thomas Scientific C000A15)
- RNAse inhibitor (Y9240L Qiagen)
- Padlock probes

Mixes

Wash-20 (Solution is stable for a week)

- 2ml of formamide
- 1ml of SSC 20x
- 7ml of ultra pure water

Wash-20 + Rnase inhibitor – Make it fresh

- 50 µl of Wash-20
- 0.5 µl of RNAse inhibitor

Padlock probe solution – Make it fresh

- 3 µl of 20X SSC
- 6 µl of formamide
- 0.6 µl of RNAse inhibitor
- 0.6 µl of Padlock probe 500nM
- 19.8 µl of H2O

Protocol

All steps must be done in a humidified chamber!

17. Add 50 µl of Wash-20 + RNAse inhibitor and incubate for 15min at room temperature.

18. Prepare the 30 µl Padlock probe solution to a final concentration of 10nM. Incubate the solution overnight at 37˚C.

Probe wash and ligation

Material

- PBS
- Wash-20
- SplintR ligase kit (NEB M0375L)
- RNAse inhibitor (Y9240L Qiagen)

Mixes

Wash-20 + Rnase inhibitor – Make it fresh

- 150 µl of Wash-20
- 1.5 µl of RNAse inhibitor

Ligase mix – Make it fresh and prepare on ice

- 2.5 µl of splintR ligase
- 5 µl of SplintR ligase buffer 10x
- 1 µl of RNAse inhibitor
- 41.5 µl Ultra pure water

Protocol

All steps must be done in a humidified chamber!

19. Wash 3 times with 50 µl of Wash-20 supplemented + RNAse. Incubate each wash 15min at 37˚C.

20. Wash with 80 µl of PBS and incubate 15min at 37˚C.

21. Wash with 80 µl of splintR ligase buffer 1X and incubate 15min at room temperature.

22. Prepare the ligase mix on ice, remove preincubation buffer, add 50 µl to the sample and incubate for 6h to overnight at 37˚C.

RCA

Material

- RCA primer
- Phi29 kit (Thermo Fisher Scientific EP0094)
- RNAse inhibitor (Y9240L Qiagen)
- Ultra pure Water
- PBS
- dNTPs 10mM (NEB N0447L)

Mixes

RCA primer hybridization mix – Make it fresh

- 200ul of Wash-20
- 1ul of RCA primer (100µM)

RCA mix -Make it fresh and prepare on ice

- 5 µl of Phi29 buffer 10x
- 1.25 µl of 10mM dNTPs
- 4 µl of Phi29 (10U/µl)
- 0.5 µl of RNAse inhibitor
- 39.25 µl of ultra pure Water

Protocol

All steps must be done in a humidified chamber!

23. Remove the ligation mix and add 50ul Wash-20 buffer for 10 min at RT.

24. Remove Wash-20 preincubation and add of 50 µl of RCA primer hybridization mix and incubate for 2hr at 37˚C.

25. Remove the primer mix and add 80 µl of Wash-20 incubated for 20min at 37˚C.

26. Remove the wash and add 80 µl of PBS incubated for 15min at 37˚C.

27. Remove the PBS and add 80 µl of 1X Phi-29 buffer incubated for 15min at RT. Switch the incubator to 30˚C.

28. Prepare the RCA mix on ice, remove preincubation buffer, add 50 µl to the sample and incubate overnight at 30˚C.

29. Remove RCA mix and add 80 µl of PBS to terminate the reaction. From this point the samples is stable for at least 3 months at 4 ˚C (probably more but we did not test it).

Probe detection through manual fluidic exchange

Material

- Ultra pure Water
- Formamide (Sigma Aldrich F7503-4L)
- 20X SSC (Thomas Scientific C000A15)
- Dapi Sigma Aldrich S40
- HW dextran sulfate 50% (Sigma Aldrich S4031)
- Wash-20
- Fluorescent probes

Mixes per N rounds

Wash-20-HWDS-Dapi

- N x 300 µl of Wash-20
- N x 0.15 µl of Dapi
- N x 3 µl of HW dextran sulfate 50%

Stripping solution

- N x 400 µl of formamide
- N x 12.5 µl of 20X SSC
- N x 87.5 µl of Ultra pure H2O

N x Probe solutions

- 30 µl of Wash-20
- 0.15 µl of fluorescent probe A 100 µM
- 0.15 µl of fluorescent probe B 100 µM
- 0.15 µl of fluorescent probe C 100 µM

Protocol

30. Prepare Wash-20-HWDS-Dapi, stripping solution and all probe solutions. Protect the Wash-20-HWDS-Dapi and fluorescent probe solutions from light.

31. Replace PBS with 150ul of wash-20 and wait for 1min. Remove solution, add 30 µl of first probe solution and wait for 15min at room temp (longer incubation does not affect the staining neither the probe stripping).

    Remove solution and add 100 µl of Wash-20
    Wait for 1 minutes
    Remove solution and add 100 µl of Wash-20
    Wait for 1 minute
    Remove solution and add 100 µl of Wash-20
    Wait for 1 minute
    Remove solution and add 100 µl of Wash-20
    Wait for 1 minute
    Remove solution and add 150 µl of Wash-20
    Wait for 3 minutes

    Image the sample

32. Follow these steps for each round of detection
    a. <u>Probe stripping</u>
       Remove solution and add 150 µl of stripping buffer
       Wait for 30 seconds
       Remove solution and add 150 µl of stripping buffer
       Wait for 30 seconds
       Remove solution and add 150 µl of stripping buffer
       Wait for 5 minutes
    b. <u>Tissue blocking and dapi</u>
       Remove solution and add 150 µl of Wash-20-HWDS-Dapi
       Wait for 30 seconds
       Remove solution and add 150 µl of Wash-20-HWDS-Dapi
       Wait for 3 minutes
    c. <u>Pre incubation</u>
       Remove solution and add 150 µl of Wash-20
       wait for 30 seconds
       Remove solution and add 150 µl of Wash-20
       Wait for 1 minute
    d. <u>Probe incubation</u>
       Remove solution and add 30 µl of probe solution
       Wait for 15 minutes
    e. <u>Probe wash</u>
       Remove solution and add add 150 µl of Wash-20

Wait for 1 minutes
Remove solution and add 150 µl of Wash-20
Wait for 1 minute
Remove solution and add 150 µl of Wash-20
Wait for 1 minute
Remove solution and add 150 µl of Wash-20
Wait for 1 minute
Remove solution and add 150 µl of Wash-20
Wait for 3 minutes

f. Imaging
Variable time

Probe detection through automated fluidic exchange

Material

- Ultra pure Water
- Formamide (Sigma Aldrich F7503-4L)
- 20X SSC (Thomas Scientific C000A15)
- Dapi Sigma Aldrich S40
- HW dextran sulfate 50% (Sigma Aldrich S4031)
- Fluorescent probes

Mixes

Wash-20
- 50 ml of formamide
- 25 ml of 20X SSC
- 175 ml of ultra pure water

Wash-20-HWDS-Dapi
- 65 ml of Wash-20
- 16.25 µl of Dapi
- 650 µl of HW dextran sulfate 50%

Stripping solution
- 56 ml of formamide
- 1.75 ml of 20X SSC
- 12.25 ml of Ultra pure H2O

N x Probe solutions
- 1 ml of Wash-20
- 3 µl of fluorescent probe A 100 µM
- 3 µl of fluorescent probe B 100 µM
- 3 µl of fluorescent probe C 100 µM

Protocol

33. Prepare Wash-20, Wash-20-HWDS-Dapi, stripping solution and all probe solutions. Protect the Wash-20-HWDS-Dapi and fluorescent probe solutions from light.

34. ▲CAUTION Remove the hydrophobic barrier using a sharp blade (the glass slide is very fragile and can break if not handle correctly). Place carefully the sample in the FCS2 flow cell and close the flow cell according to the manufacturer instructions. Connect separate tubing to each outlet of the flow cell and introduce 1ml of Wash20-DSS-Dapi with a syringe. Immediately close the tubing circuit, by connecting the same tubing on both side of the flow cell and incubate the solution for 10 minutes at room temperature.

35. introduce 3ml of Wash-20 and incubate for 5minutes at room temperature. After incubation, flow 3ml of Wash-20 and close the tubing of the flow cell, to maintain Wash-20 in the flow cell.

36. Optional: You can manually add the first probe set to the sample

    Add 500 µl of probe solution into the flow cell and incubate for at least 25min at room temp (longer incubation does not affect the staining neither the probe stripping).
    Add 2ml of Wash-20
    Wait for 3 minutes
    Add 1ml of Wash-20
    Wait for 1 minute
    Add 1ml of Wash-20
    Wait for 1 minute
    Add 1ml of Wash-20
    Wait for 1 minute
    Add 1ml of Wash-20
    Wait for 1 minute
    Add 2ml of Wash-20
    Wait for 1 minute

37. Connect all tubing from the valve controller to the probe, blocking, wash and stripping solutions. The first valve controller receives the probe mixes (one mix per cycle), and its outlet is connected to the inlet of the second rotary valve. Additionally, the inlets of the second controller valve are connected to the stripping solution, Wash-20-HWDS-Dapi and wash-20. The outlet of the second rotary valves is connected to the flow cell. The peristaltic pump is placed downstream of the flow cell and controls the flow rate at 0.3 ml/min for the probe mix buffer and 1 ml/min for the other buffers.
    To prevent the formation of bubbles, we recommend plugging tubes into all empty ports on the valve controller and connect those tubes to Wash-20.

38. Prime all tubes in this order:
    a. 200 µl of fluorescent probe tubes
    b. 200 µl of all filler tubes plug on the empty ports of the valve controller.
    c. 1ml of stripping buffer
    d. 1ml of Wash-20-HWDS-Dapi
    e. 2ml of Wash-20

    Once all tubes are primed, visually inspect that all tubes are bubble free, and connect the outlet of the valve controller to the flow cell. Flow 1ml of Wash-20 and image the round 0 from the manual probe hybridization.

39. Synchronize the automated fluidic exchange with the image acquisition using a mouse autoclicker or time series from the microscope software control.

40. Run the Sequential hybridization with the following program:
    Timing ~47minutes per cycles (without imaging time)

    a. <u>Probe stripping</u>
       2.5ml of stripping buffer
       Wait for 30 seconds
       1ml of stripping buffer
       Wait for 30 seconds
       2.5ml of stripping buffer
       Wait for 5 minutes
    b. <u>Tissue blocking and dapi</u>
       2.5ml of Wash-20-HWDS-Dapi
       Wait for 30 seconds
       2.5ml of Wash-20-HWDS-Dapi
       Wait for 3 minutes
    c. <u>Pre incubation</u>
       2.5ml of Wash-20
       wait for 30 seconds
       2.5ml of Wash-20
       Wait for 2 minutes
    d. <u>Probe incubation</u>
       600 µl of probe solution
       Wait for 25 minutes
    e. <u>Probe wash</u>
       Add 2ml of Wash-20
       Wait for 1 minutes
       Add 1ml of Wash-20
       Wait for 1 minute
       Add 1ml of Wash-20
       Wait for 1 minute
       Add 1ml of Wash-20
       Wait for 1 minute

Add 1ml of Wash-20
Wait for 1 minute
Add 2ml of Wash-20
Wait for 1 minute

f. Imaging
Variable time