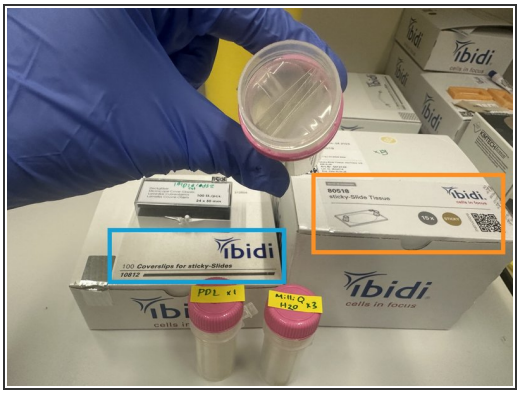
Fluigent Aria 0: Sample Preparation

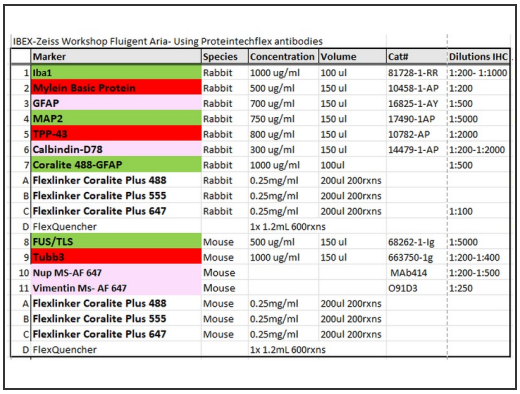
This guide describes how to prepare tissue samples to mount on an Ibidi Sticky slide to load on the Fluigent Aria.

Written By: z mbstaff



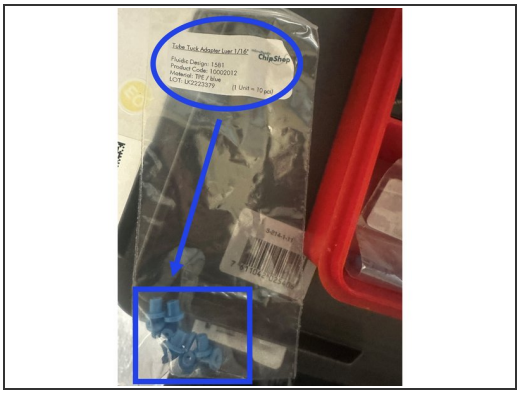
Step 1 — Required Materials





iBEX-Zeiss Workshop Fluigent Aria- Using Proteintechflex antibodies

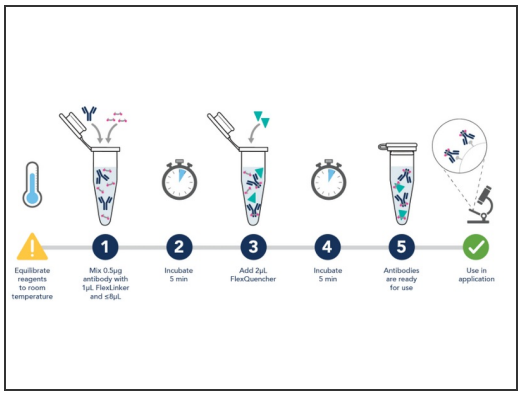
	Marker	Species	Concentration	Volume	Cat#	Dilutions IHC
1	Iba1	Rabbit	1000 ug/ml	100 ul	81728-1-RR	1:200- 1:1000
2	Mylein Basic Protein	Rabbit	500 ug/ml	150 ul	10458-1-AP	1:200
3	GFAP	Rabbit	700 ug/ml	150 ul	16825-1-AY	1:500
4	MAP2	Rabbit	750 ug/ml	150 ul	17490-1AP	1:5000
5	TPP-43	Rabbit	800 ug/ml	150 ul	10782-AP	1:2000
6	Calbindin-D78	Rabbit	300 ug/ml	150 ul	14479-1-AP	1:200-1:2000
7	Coralite 488-GFAP	Rabbit	1000 ug/ml	100ul		1:500
A	Flexlinker Coralite Plus 488	Rabbit	0.25mg/ml	200ul 200rxns		
B	Flexlinker Coralite Plus 555	Rabbit	0.25mg/ml	200ul 200rxns		
C	Flexlinker Coralite Plus 647	Rabbit	0.25mg/ml	200ul 200rxns		1:100
D	FlexQuencher		1x 1.2mL 600rxns			
8	FUS/TLS	Mouse	500 ug/ml	150 ul	68262-1-Ig	1:5000
9	Tubb3	Mouse	1000 ug/ml	150 ul	663750-1g	1:200-1:400
10	Nup MS-AF 647	Mouse			MAb414	1:200-1:500
11	Vimentin Ms- AF 647	Mouse			O91D3	1:250
A	Flexlinker Coralite Plus 488	Mouse	0.25mg/ml	200ul 200rxns		
B	Flexlinker Coralite Plus 555	Mouse	0.25mg/ml	200ul 200rxns		
C	Flexlinker Coralite Plus 647	Mouse	0.25mg/ml	200ul 200rxns		
D	FlexQuencher		1x 1.2mL 600rxns			

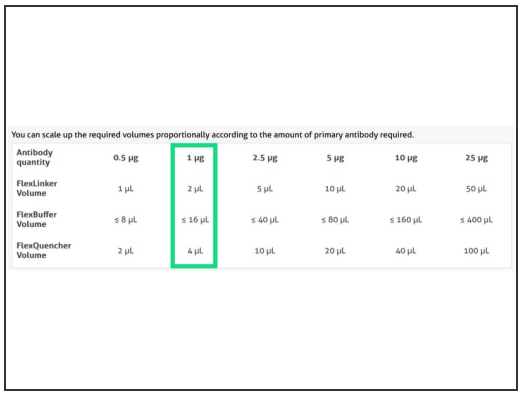


ⓘ Catalog Numbers for Ordering Reagents:

- Proteintech FlexAble 2.0 Kit and Antibodies
- Ibidi Coverslips (cat# 10812)
- Coating Reagents- Chrome Alum Gelatin (Newcomer Supply, cat no. 1033A USA) *important for tissue adherence for multiplex cycles, for adherent cells can omit. Substitution Poly-D-Lysine (cat#P6407 Sigma) or Poly-L-Lysine (cat# P8920 Sigma 0.1% w/v) coating also possible
- Ibidi Sticky Slides for Tissue (cat# 80518)
- 15mL Falcon Tubes (cat# 10773501) Thermofisher. Important to match thread size of the Aria pump.
- Connectors: https://www.microfluidic-chipshop.com/ Tube Tuck Adapter Luer 1/16" Fluidic Design: 1581

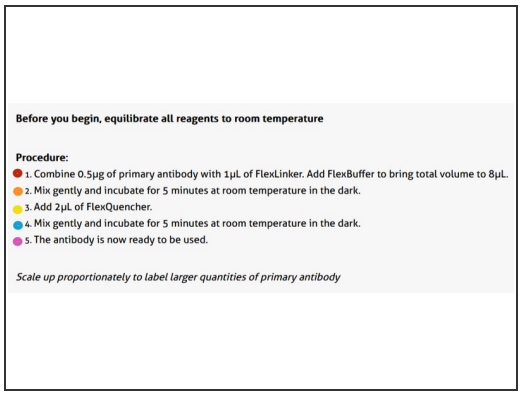
Step 2 — Prepare Antibodies: Conjugation with Proteintech FlexAble 2.0 Kit





You can scale up the required volumes proportionally according to the amount of primary antibody required.

Antibody quantity	0.5 µg	1 µg	2.5 µg	5 µg	10 µg	25 µg
FlexLinker Volume	1 µL	2 µL	5 µL	10 µL	20 µL	50 µL
FlexBuffer Volume	≤ 8 µL	≤ 16 µL	≤ 40 µL	≤ 80 µL	≤ 160 µL	≤ 400 µL
FlexQuencher Volume	2 µL	4 µL	10 µL	20 µL	40 µL	100 µL



Before you begin, equilibrate all reagents to room temperature

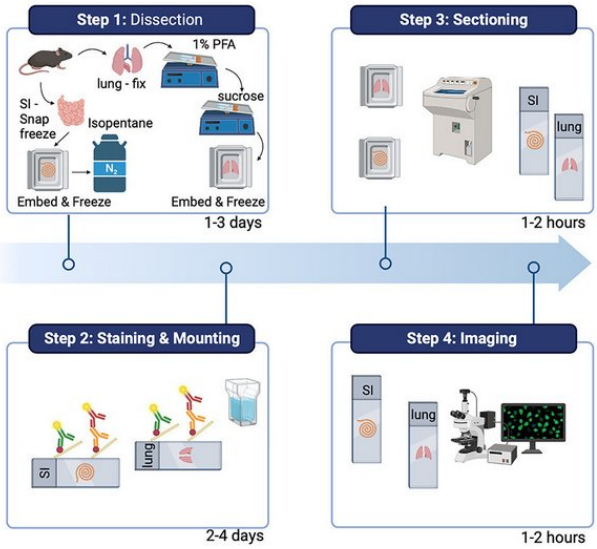
Procedure:

1. Combine 0.5µg of primary antibody with 1µL of FlexLinker. Add FlexBuffer to bring total volume to 8µL.
2. Mix gently and incubate for 5 minutes at room temperature in the dark.
3. Add 2µL of FlexQuencher.
4. Mix gently and incubate for 5 minutes at room temperature in the dark.
5. The antibody is now ready to be used.

Scale up proportionately to label larger quantities of primary antibody

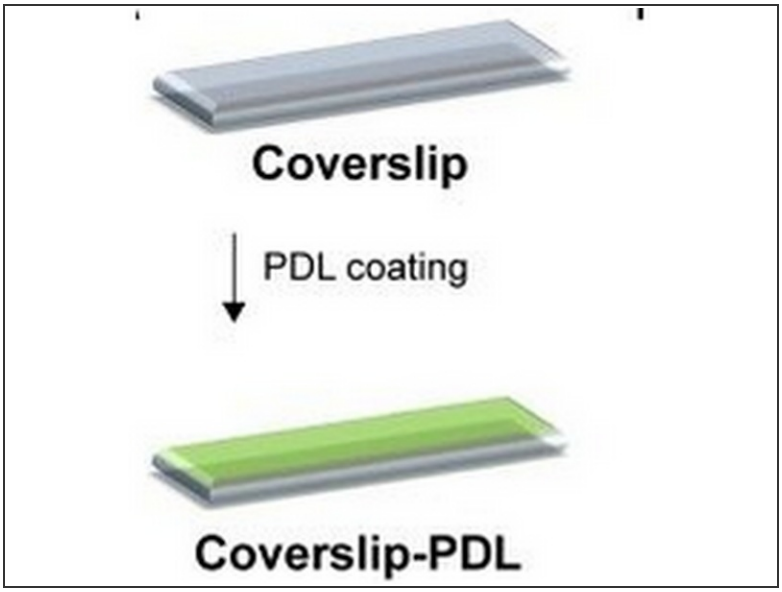
- Proteintech- Antibody Labeling with FlexAble 2.0 Kit. **Use 1ug of antibody**: Proteintech FlexAble calculator
- Assign Antibody to Flexlinker Coralite Plus Flurophore (i.e. 488, 555, or 647).
- 1. Combine 1.0 ug of primary antibody (i.e, 1.4uL of GFAP 700ug/mL Cat#16825-1AY) with 2ul of FlexLinker Coralite Plus 555. Add 12.6uL of flexBuffer to bring total volume to 16uL.
- 2. Mix gently and icubate for 5 minutes at room temperature in the dark (in opaque microcentrifuge tubes).
- 3. Add 4uL of FlexQuencher.
- 4. Mix gently and incubate for 5 minutes at room temperature in the dark.
- The Antibody is now ready to use- Store at 4 degrees.

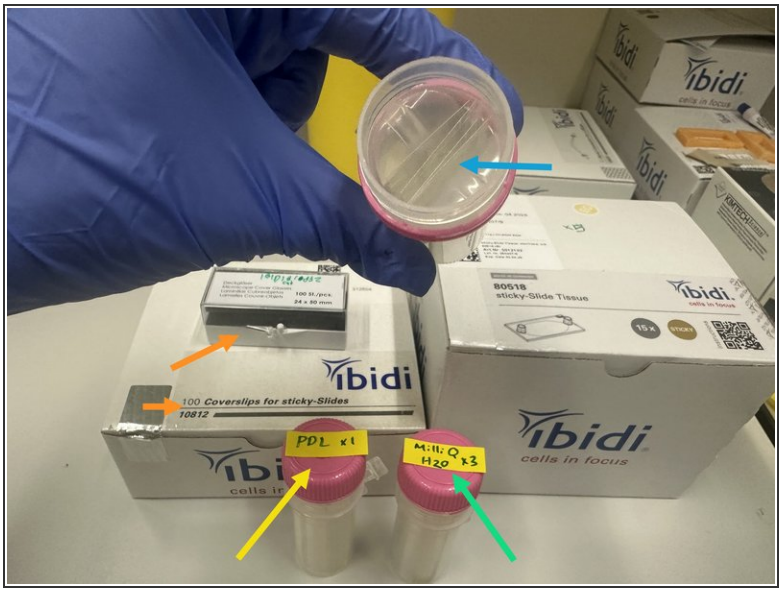
Step 3 — Tissue Processing



- Take Protocol of Choice: One here or steps below.
- 1. Perfusion with PBS followed by 4%PFA, until tissue appears white.
- 2. Post Fix for 1hr in 4% PFA (adjust time to tissue size).
- 3. Rinse 3x with PBS, and soak in Sucrose 30% overnight.
- 4. Fill mold with OCT, and position tissue in mold mindful of orientation for future cryosectioning.
- 5. Freeze in in finely crushed dry ice, wait for OCT to become opaque, before moving and storing at -80 before cyrosectioning.
- Always acclimate block to cryostat temperature before sectioning.

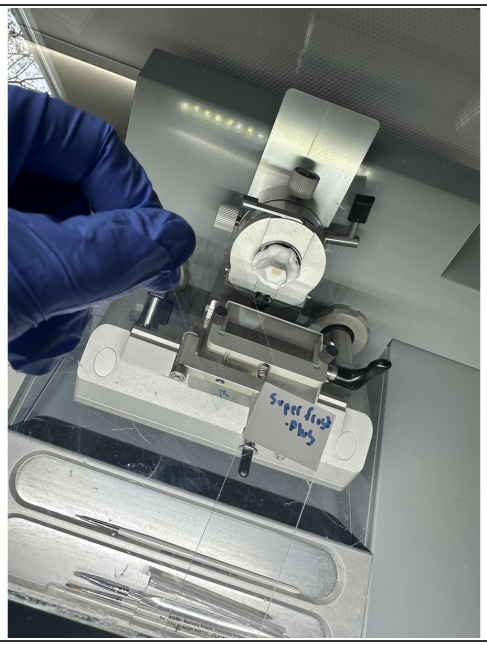
Step 4 — Prepare Coverslips





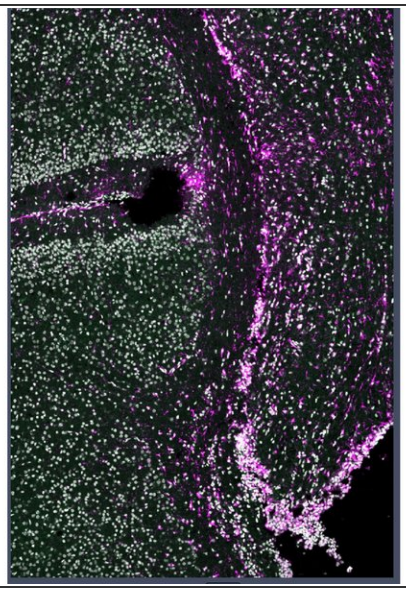
- IBEX- Recommended to coat in Chrome Alum Gelatin
- Substitution Poly-D-Lysine (cat#P6407 Sigma) or Poly-L-Lysine (cat# P8920 Sigma 0.1% w/v) coating also possible
- Poly D Lysine (PDL) Sigma Aldrich- P6407-5mg- substitute for original publication Chrome Alum Gelatin (Newcomer Supply, cat no. 1033A USA) not available in EU or CH. *important for tissue adherence for multiplex cycles, for adherent cells can omit.
- 1. Ibidi Glass Coverslips for sticky slides.Cat# 10812 #1.5H Glass Coverslip 25mm x 75 mm
- 2. Coat surface (marking coated side) or submerge in coating. Remove excess liquid (can be used for coating subsequent coverslips)
- 3. Rinse 3x with dH2O
- 4. Dry for at least 2 hours
- 5. Ready to section onto. Do not freeze once coated.

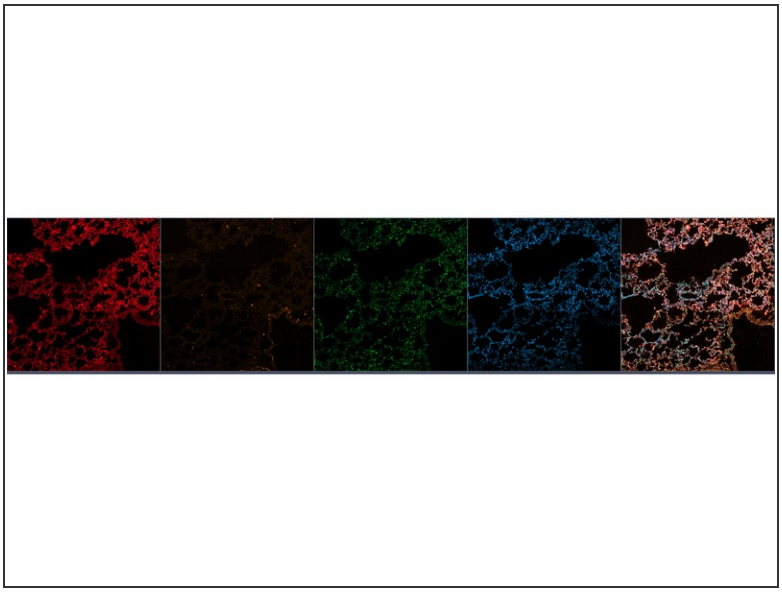
Step 5 — Collect Cryosections



- 1. Section at 10 microns. Or ideal section thickness for tissue and microscope of choice acquisition.
- 2. Collect sections onto PDL coated (or alternative coating) Ibidi coverslips.
- 3. Collect extra region of interest sections on several superfrost plus slides, for validating antibody panel prior to multiplexing experiment.
- 4. Store at 4 degrees do not freeze until ready for mounting on Ibidi Sticky slides.

Step 6 — Optimize Antibody Dilutions on Sample Tissue with IHC





- 1. Prepare Blocking Buffer from 1X PBS (Gibco, cat no. 10010-023) containing 0.3% Triton-X-100 (Millipore Sigma, cat no. 93443), 1% Bovine Serum Albumin (Millipore Sigma, cat no. A1933) and

⚠ *1:100 dilution of Human (BD Biosciences, cat no. 564219) or Mouse (BD Biosciences, cat no. 553141) Fe-block or TrueStain FcX (BioLegend, cat no. 101320). • *ZMB omit the Fe in the block.

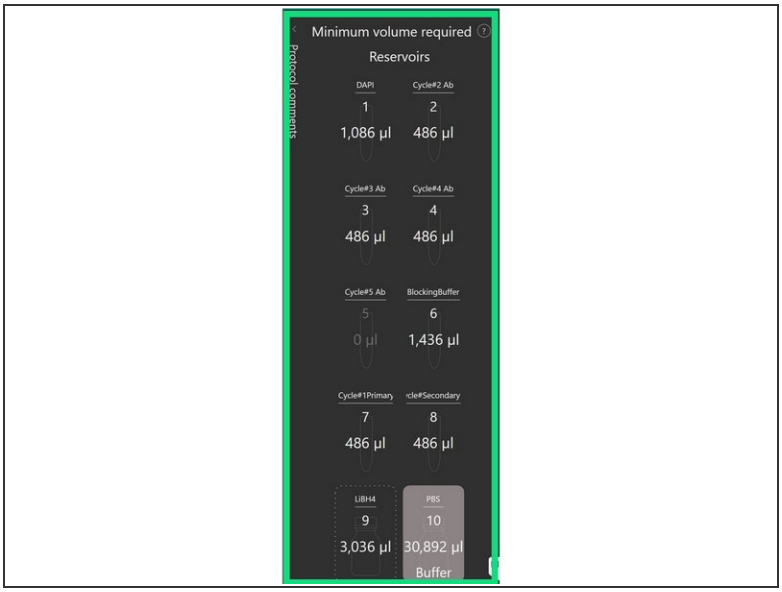
- 2. Prepare Antibody Dilutant from 1X PBS, with 0.1% BSA.
- 3. Prepare Antibodies with suggested dilutions.
- 4. Block test sections on Superfrost Plus slides, in humidified chamber for 20min-1hr.
- 5. Leave Antibody on slide for 2hrs at RT, or overnight at 4 degrees.
- 6. Test Exposure settings and verify that antibody dilutions are correct for your tissue of interest.

ⓘ Once confirmed correct staining of single cycles from your panel on slide use same dilutions for your Fluigent Aria Multiplexing Protocol in combination with other cycles.

Step 7 — Prepare Antibody Panel, Buffers, and other Reagnets for Multiplexing Experiment

IBEX-Zeiss Workshop Fluigent Aria- Using Proteintechflex antibodies

Plex	Marker	Cycle	Dilution	Species	Secondary	Dilution
1	Tomm20	1	1:500	rabbit	donkey anti rabbit AF488	1:500
2	Reelin	1	1:250	goat	donkey anti goat AF555	1:500
3	Vimentin-AF647	1	1:200			
Plex	Marker	Cycle	Dilution	Species	Secondary	Dilution
4	Iba1- Coralite Plus 488	2	1:200	rabbit	-	
5	GFAP- Coralite Plus 555	2	1:500	rabbit	-	
6	Calbindin-D78- Coralite Plus 647	4	1:100	rabbit	-	
Plex	Marker	Cycle	Dilution	Species	Secondary	Dilution
7	Fus/TLS- Coralite Plus 488	3	1:200	mouse	-	
8	Tubb3- Coralite Plus 555	3	1:100	mouse	-	
9	Nup-AF647	3	1:200	rabbit	-	
Plex	Marker	Cycle	Dilution	Species	Secondary	Dilution
10	GFAP- Coralite Plus 488	4	1:500	rabbit	-	
11	TPD-43- Coralite Plus 555	4	1:100	rabbit	-	
12	Myelin- Coralite Plus 647	4	1:100	rabbit	-	

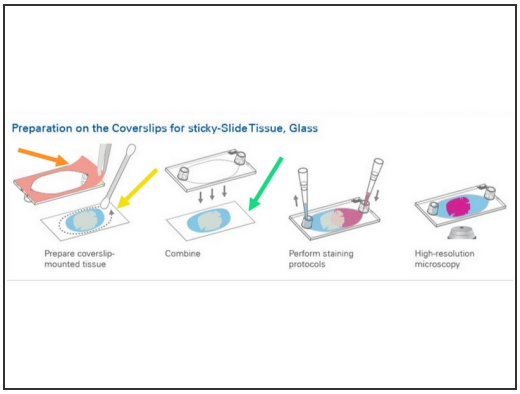


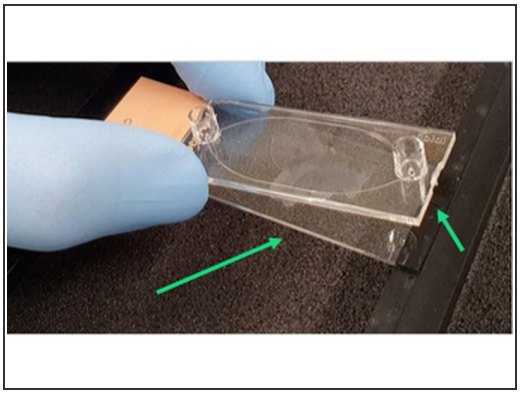
- Example Panel for IBEX Multiplexing Experiment on Neuronal Tissue
- Indirect Labeling- Primary and Secondary Antibodies can only be used in Cycle 1 in IBEX
- Direct Labeled Antibodies- Conjugated Primaries can be used in all other Cycles (Cycle 2- Cycle# n) or omit indirect labeled in Cycle 1 and use conjugated for all IBEX cycles.
- Load Reagents (1-8) in 15mL Falcons at correct dilutions and add freshly filtered LiBH4 and PBS to the 100mL glass bottles in reservoirs #9 and #10 respectively, label in Aria software.
- Prepare the following buffers: 1X PBS, Blocking Buffer (1% BSA in 1X PBS), Antibody Dilutient (0.1%BSA in 1XPBS), Lithium Borohydrate (LiBH4_
- Critical: When you prepared LiBH4 in diH20 do so in a hood and make a 1 mg/ml solution at least 5ml at a time but never more than 10mL. Leave to~incubate at room temperature for 10min, after gentle mixing. Pass the solution through a 0.22 µm syringe filter to remove any impurities.

⚠ Caution: Perform the LiBH4 preparation steps in a chemical hood with appropriate PPE. The reaction can generate hydrogen gas, which is flammable. To avoid flames, always work with small amounts (<10 mg) of Li8H4.

Store LiBH4 in a sealed container with desiccant and use a new vial of LiBH4 no later than 4 weeks after opening original container.

Step 8 — Mount coverslip on Ibidi Sticky slide



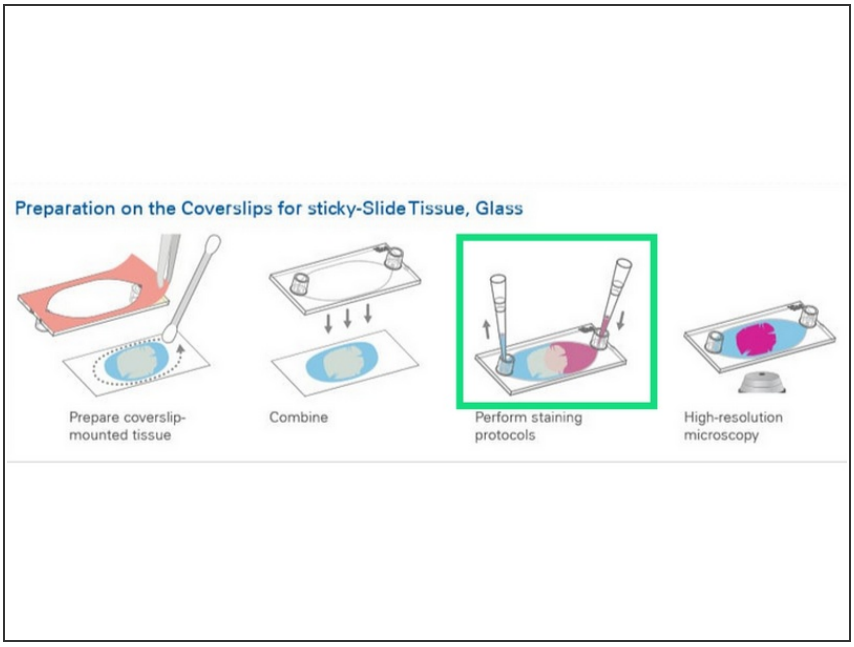




- 1. Pull away Ibidi Sticky slide film to reveal sticky coating
- 2. Clean excess tissue or folded sections away and make dry surface for applying sticky slide to glass coverslip
- 3. Align Ibidi 25mm x 75mm coverslip (cat# 10812) with Ibidi sticky slide (cat# 80518) carefully.

⚠ Do not block ports with tissue and carefully align so no over or under hangs of the glass coverslip.

Step 9 — Prefill Chamber with PBS

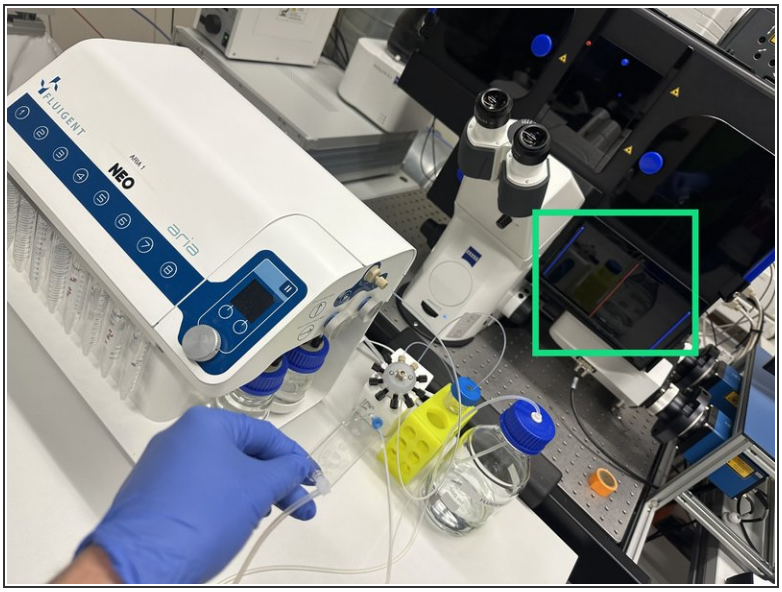


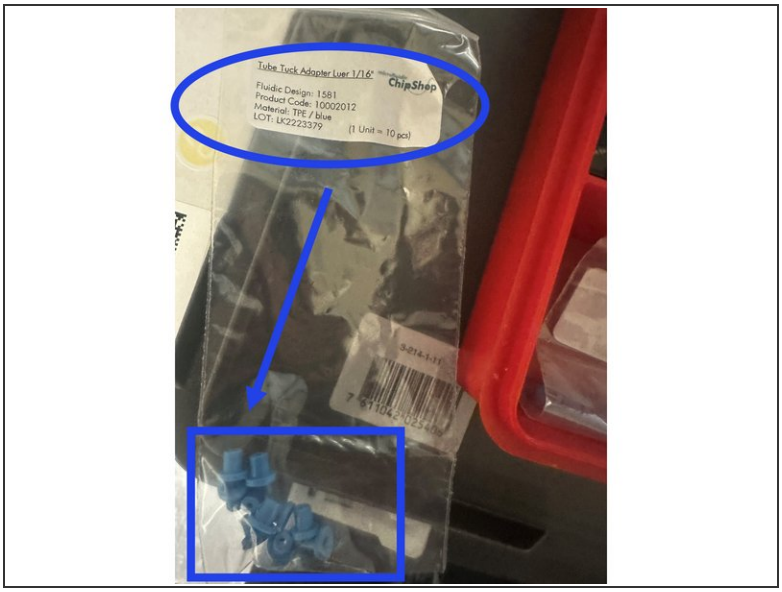
- Fill Chamber with PBS or TBS and avoid trapping air bubbles.

ⓘ Give time for OCT from sections to dissolve and tissue to acclimate to buffer.

⚠ Tap away any bubbles in chamber before proceeding with Multiplex Experiment using Fluigent Aria Pump

Step 10 — Attach Ibidi Carrier to Aria Tubing



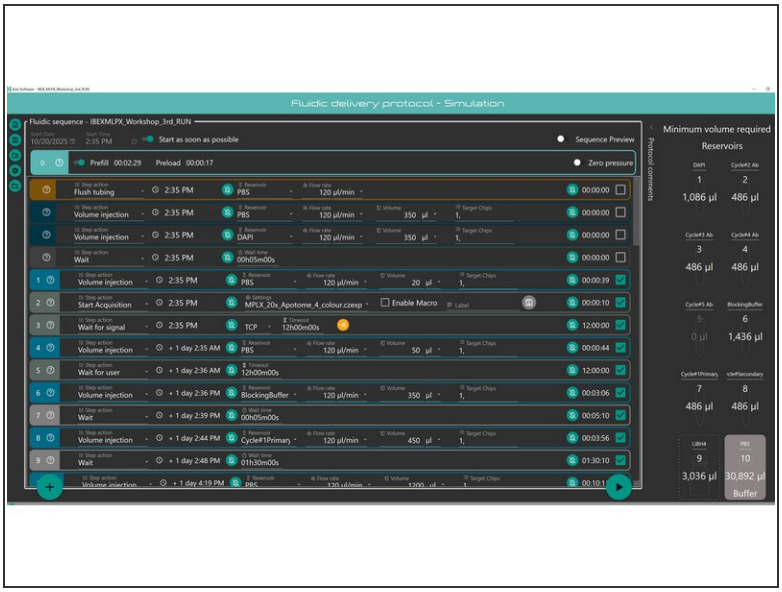


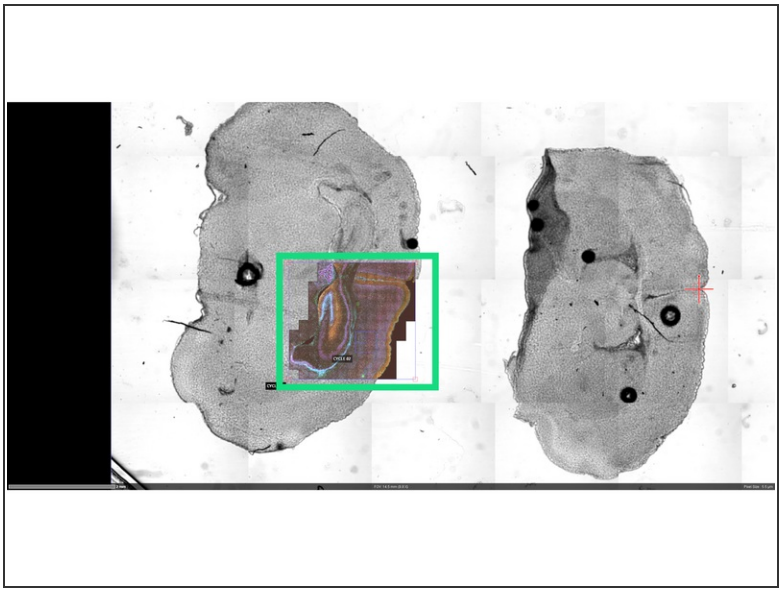
- 1. Attach blue connector to inlet (ChipShop catalog #1581 Tube Tuck Adapter Luer 1/16")
- 2. Attach elbow clear connector to waste output outlet

⚠ Can manually use Fluigent Aria pump (not with software, but with Aria control panel on pump) to load fresh buffer and confirm no bubbles or leaks.

- 3. Feed tubing through microscope door
- 4. Mount sticky slide-tissue coverslip into slide carrier to load in the microscope, within the microscope.
- 5. Check that no tubing interferes with stage movement or acquisition.
- 6. Set up slide overview and acquisition settings as in this guide: Fluigent Aria 3: Combine microfluidics with ZEISS microscopes

Step 11 — Run Personalized Aria Protocol and Microscope Multiplexing Acquistion

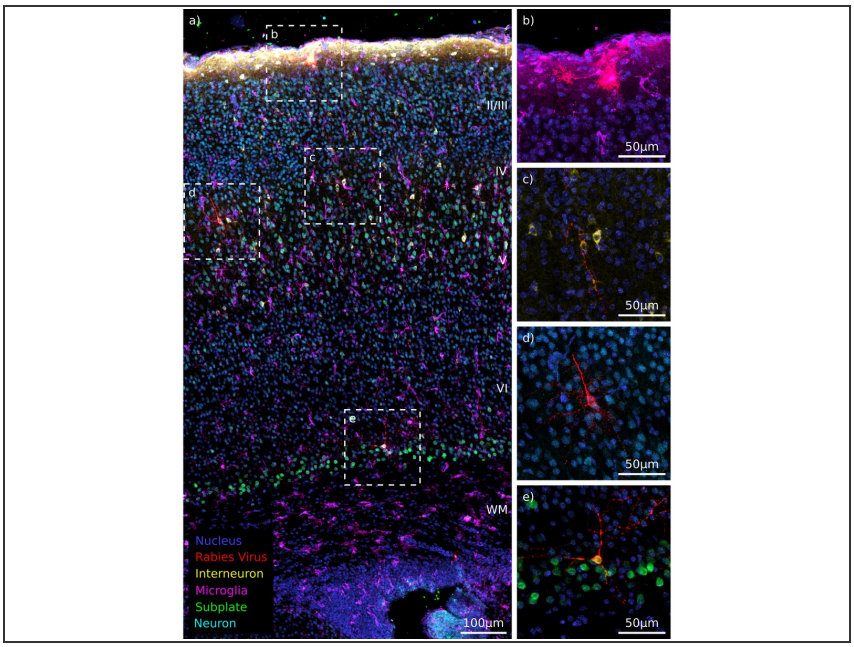




1. Setup Aria Protocol for Multiplexing Protocol of Choice- with your Reagents.

- Guide here for writing an Aria Protocol- Fluigent Aria 2: How to write an Aria Sequence
- Guide here for starting the Aria Pump- Fluigent Aria 1: Startup
- Guide here for Aria to Zeiss Microscope- Fluigent Aria 3: Combine microfluidics with Zeiss Microscopes
- Set region of interest of your tissue section

Step 12 — Panel Redesign from results



- Evaluate results from your run.
- Optimize. 1. Dilutions of Antibodies 2. Placement of tissue 3. Positioning of Antibodies in Cycles. 4. Bleaching strategy (IBEX) or Elution strategy (4i) of antibodies from each cycle
- ⓘ Strategy to Run a Full Multiplex SeqIF
- Run a real Multiplexing experiment!