RNAScope HiPlex Experiment Checklist for Fixed FROZEN Samples					
Method: RNAscope HiPlex					
Experiment Name			Sample Info		
DATE:					
Sample Preparation Steps					
	Prechill 4% PFA on ice or in 4 ºC fridge				
1	OCT Removal	1X PBS	5min	RT	Coplin Jars
2	Drying	*NA*	30min	60°C	
3	Post-fixation on ice	4% PFA, cold	15min	4°C	
4	Dehydration				
		a 50% EtOH (50 ml)	5min	RT	
		b 70% EtOH (50 ml)	5min	RT	
		c 100% EtOH (50 ml)	5min	RT	
		d 100% EtOH (50 ml)	5min	RT	
		e 100% EtOH (50 ml)	5min	RT	
	Slides may be stored in 100% Ethanol at -20ºC for 1 week				
Target Retrieval Using Temperature Controlled Heater					
	Prewarm the Target Retrieval Buffer to >= 99ºC, but do not boil for > 15 min before use				In Staining Rack
5	Acclimate to 99°C	DW preheated in steamer	Briefly	99°C	
6	Target Retrieval	Retrieval Buffer (kit)	5 min	99°C	
7	Rinse	DW	15 sec	RT	
8	Dehydration	100% EtOH	3 min	RT	
9	Drying		5 min	60°C	Laying Flat
10	Create hydrophobic barrier, let barrier dry completely	*NA*	5min	RT	
	Place a humidifying paper in humidity control tray and wet completely with distilled water				
11	Load slides into ACD EZ-Batch Slide Holder	*NA*	Briefly	RT	
12	Protease Digestion	Protease III (kit)	15-30 min	40°C	
13	Rinse **X2**	PBS	2 min	RT	
HiPlex Protocol					
Round 1	**Prepare 3 L 1 X Wash Buffer, 4 X SSC, 1 L 0.5% PBST**				
	Warm up RNAscope HiPlex probe stocks and diluent at 40 ºC for about 10 min				
	Prepare target probe with warmed HiPlex probe stocks and diluent, mix well and cool to RT before use				
	Set HybEZOven and prepared humidiity control tray at 40ºC				
	Set RNAscpe HiPlex Amp 1-3 and RNAscope HiPlex Fluoro T1-T4 V2 reagents at RT				
	Bring RNAscope HiPlex FFPE Reagent to RT, Prepare 2.5-5% FFPE reagent by using 1:40-1:20 ratio of FFPE reagent to 4X SSC				
14	**Probe Incubation**	Diluted Probes	2 hrs	40°C	
15	Wash **X2**	Wash Buffer	2 min	RT	
16	Amplification	(kit reagents)			
		a) AMP1	30 min	40°C	
		b) *WASH Buffer*	*2x2 min*	*RT*	
		c) AMP2	30 min	40°C	
		d) *WASH Buffer*	*2x2 min*	*RT*	
		e) AMP3	30 min	40°C	
		f) *WASH Buffer*	*2x2 min*	*RT*	
17	FFPE Reagent (optional)	diluted 2.5-5% in 4 X SSC	30 min	RT	
18	Wash **X2** (optional, only FFPE Reagent)	Wash Buffer	2 min	RT	
19	RNAscope HiPlex Fluoro T1-T4 V2	Kit Reagent	15 min	40°C	
20	Wash **X2**	Wash Buffer	2 min	RT	
21	Nuclear Staining	DAPI (Kit Reagent)	**30 SEC**	**RT**	
22	Cover Glass	Prolong Gold			
	Store slides in the dark at 2-8 ºC				
23	Image slides for round 1	Slide Scanner	6 hrs		
Round 2	**Prepare 4 X SSC, 1 L 0.5% PBST**				
	Set RNAscpe HiPlex Amp 1-3 and RNAscope HiPlex Fluoro T5-T8 V2 reagents and FFPE reagent (Optional) at RT				
	Prepare 10% cleaving solution v1 by diluting with 4X SSC				
24	Remove Coverslips: Soak slides until the cover slips fall off the slides	4 X SSC	1-2 hrs	RT	
25	Rinse	4 X SSC	briefly	RT	
26	Cleave the T1-T4 V2 fluorophores	10% diluted in 4X SSC	15 min	RT	
27	Wash with PBST **X2**	0.5% PBST	2 min	RT	
28	Cleave the T1-T4 V2 fluorophores	10% diluted in 4X SSC	15 min	RT	
29	Wash with PBST **X2**	0.5% PBST	2 min	RT	
30	FFPE Reagent (optional)	diluted 2.5-5% in 4 X SSC	30 min	RT	
31	Wash **X2** (optional, only for FFPE Reagent)	Wash Buffer	2 min	RT	
32	RNAscope HiPlex Fluoro T5-T8 V2	Kit Reagent	15 min	40°C	
33	Wash **X2**	Wash Buffer	2 min	RT	
34	Nuclear Staining	DAPI (Kit Reagent)	**30 SEC**	**RT**	
35	Cover Glass	Prolong Gold			
	Store slides in the dark at 2-8 ºC				
36	Image slides for round 2	Slide Scanner	6 hrs		
Round 3	**Set RNAscpe HiPlex Amp 1-3 and RNAscope HiPlex Fluoro T9-T12 reagents and FFPE reagent (Optional) at RT**				
	Prepare 10% cleaving solution v1 by diluting with 4X SSC				
37	Remove Coverslips: Soak slides until the cover slips fall off the slides	4 X SSC	1-2 hrs	RT	
38	Rinse	4 X SSC	briefly	RT	
39	Cleave the T5-T8 V2 fluorophores	10% diluted in 4X SSC	15 min	RT	
40	Wash with PBST **X2**	0.5% PBST	2 min	RT	
41	Cleave the T5-T8 V2 fluorophores	10% diluted in 4X SSC	15 min	RT	
42	Wash with PBST **X2**	0.5% PBST	2 min	RT	
43	FFPE Reagent (optional)	diluted 2.5-5% in 4 X SSC	30 min	RT	
44	Wash **X2** (optional, only for FFPE Reagent)	Wash Buffer	2 min	RT	
45	RNAscope HiPlex Fluoro T9-T12 V2	Kit Reagent	15 min	40°C	
46	Wash **X2**	Wash Buffer	2 min	RT	
47	Nuclear Staining	DAPI (Kit Reagent)	**30 SEC**	**RT**	

48	Cover Glass	Prolong Gold	
	Store slides in the dark at 2-8 ºC		
49	Image slide for round 3	Slide Scanner	6 hrs

Fixed Frozen Tissues:

Fix samples
1. If needed, perfuse tissue with freshly prepared 4% paraformaldehyde (PFA) in 1X PBS or go directly to Step 2.
Note: We recommend perfusing tissues with 1X PBS followed by freshly prepared 4% paraformaldehyde (PFA) in 1X PBS. For suboptimally prepared samples, you may need to adjust pretreatment conditions.
2. Dissect tissue and fix in freshly prepared 10% NBF or 4% PFA for 24 HRS at 4°C.

Freeze tissues
1. Immerse the tissue in 10% sucrose in 1X PBS at 4°C until the tissue sinks to the bottom of the container (approximately 18 HRS for brain tissue).
2. Repeat this step with 20% sucrose in 1X PBS, followed by 30% sucrose in 1X PBS, each time allowing the tissue to sink to the bottom of the container. Freeze the tissue in Optimal Cutting Temperature (OCT) embedding media with dry ice, liquid nitrogen, or 2-methyl butane, and store it in an airtight container at –80°C.

Prepare sections
1. Before tissue sectioning, equilibrate the tissue blocks at –20°C for at least 1 HR in a cryostat.
2. Section the blocks by cutting 7–15 µm thick sections. Mount the sections on SUPERFROST PLUS SLIDES. Do not place sections too close to the edges of the slide.
3. Air dry the slides for 60 –120 MIN at –20°C. Use sectioned tissue within three months. Store sections with desiccants at –80°C.