Nov 29, 2021

Targeted ExSeq -- In Situ Sequencing (Illumina Chemistry)

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dx.doi.org/10.17504/protocols.io.bgs4jwgw

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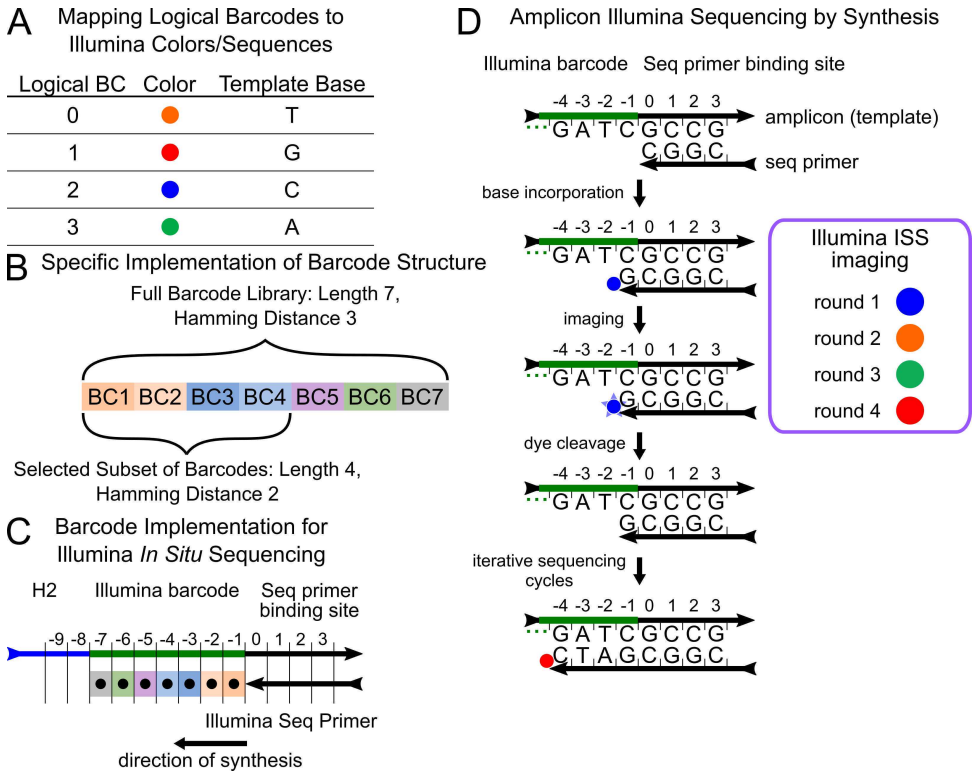
Citation: Yi Cui, Anubhav Sinha, Ed Boyden Targeted ExSeq -- In Situ Sequencing (Illumina Chemistry)ÃÂ
https://dx.doi.org/10.17504/protocols.io.bgs4jwgw



This protocol accompanies Expansion Sequencing (ExSeq), and describes the process of performing in situ sequencing with the Illumina chemistry.

The steps here are an extension of the protocol used in the original paper and are optimized for reading longer barcodes of up to seven bases. For shorter barcodes (less than four bases) in brain tissue, we recommend using the experimental protocol from the paper (with the imaging conditions as described in this protocol) as it is simpler and slightly faster.

There are two stages to this protocol: (1) sample preparation (steps 5-10), involving anchoring the sample to a glass-bottom plate and preparing the sample for sequencing; and (2) *in situ* sequencing using the Illumina sequencing-by-synthesis chemistry (steps 11-15). After *in situ* sequencing is complete, there is an optional antibody staining recovery step (step 16) if antibody staining was performed during tissue preparation.

The relationship between template bases and fluorophores is shown in **Fig. 1A**. Note that the added base (that bears the fluorophore) is the complementary base, i.e. the modified dCTP from the Illumina reagents bears the most redshifted dye. The specific length 7, Hamming distance 3 logical barcode library used in these protocols is shown in **Fig. 1B**, with the specific readout strategy for Illumina *in situ* sequencing shown in **Fig. 1C**.

The process of Illumina sequencing-by-synthesis *in situ* is shown in **Fig. 1D**. There are three key steps: an incorporation step, in which the next base of the barcode is synthesized; an imaging step, in which the sample is imaged on a spinning disk confocal microscope; and a cleavage step, in which the dye is cleaved, and the reversible terminator is unblocked, enabling the next round of synthesis to be performed.

This protocol was used to profile human metastatic breast cancer biopsies as a part of the Human Tumor Atlas Pilot Project.

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DOI
dx.doi.org/10.17504/protocols.io.bgs4jwgw

https://pubmed.ncbi.nlm.nih.gov/33509999/

Yi Cui, Anubhav Sinha, Ed Boyden 2021. Targeted ExSeq -- In Situ Sequencing (Illumina Chemistry) . **protocols.io**
https://dx.doi.org/10.17504/protocols.io.bgs4jwgw

protocol

Alon S*, Goodwin DR*, Sinha A*, Wassie AT*, Chen F*, Daugharthy ER**, Bando Y, Kajita A, Xue AG, Marrett K, Prior R, Cui Y, Payne AC, Yao CC, Suk HJ, Wang R, Yu CJ, Tillberg P, Reginato P, Pak N, Liu S, Punthambaker S, Iyer EPR, Kohman RE, Miller JA, Lein ES, Lako A, Cullen N, Rodig S, Helvie K, Abravanel DL, Wagle N, Johnson BE, Klughammer J, Slyper M, Waldman J, Jané-Valbuena J, Rozenblatt-Rosen O, Regev A; IMAXT Consortium, Church GM***+, Marblestone AH***, Boyden ES***+ (2021) Expansion Sequencing: Spatially Precise In Situ Transcriptomics in Intact Biological Systems, Science 371(6528):eaax2656. (* equal contribution, ** key contributions to early stages of project, *** equal contribution, +co-corresponding authors)

Targeted Expansion Sequencing Protocols

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expansion microscopy, in situ sequencing, expansion sequencing, targeted ExSeq, ExSeq, spatial transcriptomics, spatial omics, spatially resolved transcriptomics

protocol ,

May 23, 2020

Nov 29, 2021

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Targeted Expansion Sequencing Protocols
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This protocol follows the Tissue Preparation and Library Preparation protocols; those guidelines also apply here.

☒ Dideoxynucleoside Triphosphate Set **Millipore Sigma Catalog #03732738001**

☒ MiSeq Reagent Kit v3 (or v2) **illumina**

☒ 200 Proof Ethanol pure **Sigma Aldrich Catalog #E7023**

☒ UltraPure™ DNase/RNase-Free Distilled Water **Thermofisher Catalog #10977023**

☒ Acetic acid **Millipore Sigma Catalog #695092-100ML**

☒ Bind-silane **Millipore Sigma Catalog #GE17-1330-01**

☒ N,N,N',N'-Tetramethylethylenediamine **Sigma Catalog #T7024**

☒ Ammonium persulfate (APS) **Sigma Aldrich Catalog #A3678**

☒ Acrylamide/Bis 19:1, 40% (w/v) solution **Thermo Fisher Catalog #AM9022**

☒ Tris Base **Sigma Aldrich Catalog #648310**

☒ TetraSpeck™ Microspheres, 0.2 µm, fluorescent blue/green/orange/dark red **Thermo Fisher Catalog #T7280**

☒ PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific Catalog #AM9625**

☒ SSC (20X) RNase-free **Thermo Fisher Scientific Catalog #AM9770**

☒ Terminal Transferase - 500 units **New England Biolabs Catalog #M0315S**

☒ Standard Taq (Mg-free) Reaction Buffer Pack - 6.0 ml **New England Biolabs Catalog #B9015S**

☒ Zwittergent® 3-12 Detergent **Millipore Sigma Catalog #693015-5GM**

☒ DAPI **Thermo Fisher Scientific Catalog #62248**

Optional:

☒ MES 0.5M buffer soln. pH 6.0 **Fisher Scientific Catalog #AAJ62574AE**

☒ Streptavidin, Alexa Fluor™ 488 conjugate **Thermo Fisher Catalog #S11223**

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IMT (IMS), USM (SRE), CMS (CMS), and PR2 (PR2) are harvested from the MiSeq v3 (v2) kit.

Terminal Transferase from NEB includes Terminal Transferase Reaction Buffer Pack and CoCl2 solution.

The Taq (Mg-free) reaction buffer pack includes MgCl2.

Sequencing primer: TCTCGGGAACGCTGAAGACGGC from a DNA synthesis company at >=100 nmol synthesis scale, with HPLC purification.

Other materials:

- Basic lab wetware (pipettes, paintbrushes, lab tape, tweezers)
- Tupperware container (as in tissue preparation protocol)
- Blunt needles
- 24-well glass-bottom plate (may need larger 6-well glass-bottom plate depending on size of gel)
- 10 mm circular coverglasses (may need different size of coverglass depending on size of gel)

Equipment:

- **37 °C** and **50 °C** incubators
- Access to a spinning disk confocal microscope with 405, 488, 561, 647 and 685 nm laser lines. Alternatives are discussed in the protocol.

Please carefully read all safety datasheets for all reagents used in the protocol, and perform all steps in accordance with relevant guidelines.

:

Before starting, prepare stock solutions/reagents as described in steps 1-4 of the protocol.

Preparation 2h

1 Preparation of Sample

This protocol assumes that samples have been prepared following the Targeted ExSeq Tissue Preparation protocol, and that a sequencing library has been prepared following the Targeted ExSeq Library Preparation protocol. The padlock probes to generate the library should have been prepared following the Targeted ExSeq Probe Generation protocol.

The protocol assumes that the library has been prepared, and if the amplicons were optionally checked, that the detection oligos have been stripped off as described in the Library Preparation protocol.

2 Pooling ddNTPs

5m

The dideoxynucleotide set contains four tubes at 10 mM each for each of the dideoxynucleotides.

Combine 10 µL from each tube to form the final 2.5 mM each ddNTP pool.

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3 Preparing Sequencing Reagents from MiSeq Kit

1h

The Illumina *in situ* sequencing kit reagents are collected from MiSeq reagent kits. Both MiSeq v3 and v2 reagent kits can be used for *in situ* sequencing. The terminology below is written for the MiSeq v3, with MiSeq v2 terms in parentheses.

Three reagents are collected:
- IMT: Incorporation Mix (v2: IMS, Incorporation Mix)
- USM: Scan Mix (v2: SRE, Scan Mix)
- CMS: Cleavage Mix (v2: CMS, Cleavage Mix)

Defrost a MiSeq v3 reagent kit (either **Overnight** at **4 °C** or for **01:00:00** in a **Room temperature** water bath). Invert the kit to mix and ensure that the wells are fully melted.

Wipe a clean laboratory wipe over the foil cover of Wells 1, 2, and 4 of the MiSeq Reagent Kit.

Pierce the foil for Wells 1, 2, and 4 with a 1 mL pipette tip.

Use a transfer pipette to transfer reagents in Well 1 (IMT; v2 IMS), Well 2 (USM; v2 SRE), and Well 4 (CMS; v2 CMS) to individual 50 mL tubes.

Aliquot IMT (IMS), USM (SRE), and CMS (CMS) into 250-300 µL aliquots in microcentrifuge tubes, and store at **-20 °C** for up to one month.

In addition, also save the bottle of PR2 provided with the reagent kit.

4 Gelling Materials from ExSeq Tissue Processing Protocol

The following reagents were prepared in the Targeted ExSeq Tissue Processing Protocol and are used again in this protocol.

1. 10% TEMED
2. 10% APS

Second Re-embedding Gel 5h

5 Overview of Second Re-embedding

To minimize any movement during the multiple rounds of imaging during *in situ* sequencing, the sample gel is immobilized to a glass-bottom plate for all subsequent steps. This is accomplished by functionalizing wells of a glass-bottom plate with acryloyl groups (Step 6), and casting a second re-embedding gel to anchor the gel to the plate (Step 7).

6 Treatment of Glass-Bottom Plate with Bind-Silane

1h 30m

In this step, we treat wells of a 24-well glass-bottom plate with Bind-Silane, a silanization reagent that functionalizes the glass surface with acryloyl groups. The acryolyl groups are incorporated as monomers of the gel formed during the second re-embedding step.

Volumes can be scaled up as is appropriate for 6-well plates.

6.1 Cleaning Glass Surfaces

5m

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Wash wells briefly with ddH2O, followed by 100% ethanol.

6.2 Bind-Silane Reaction

1h 15m

Prepare Bind-Silane reaction mix, scaling up/down total volume as needed.

A	B
Reagent	**Volume**
Bind-Silane Reagent	10 µL
Ethanol	4000 µL
Glacial Acetic Acid	100 µL
Water	890 µL
Total	5000 µL

Bind-Silane reaction mix

Treat wells of glass-bottom plate for **00:45:00** at **Room temperature** .

Remove bind-silane reaction mix and allow well to dry.

Wash twice briefly with 100% ethanol, then remove ethanol and allow wells to dry completely before use.

Directly proceed to casting second re-embedding gel.

7 Casting Second Re-embedding Gel

3h

This gelling step is similar to the first re-embedding step from the tissue preparation protocol, but does not have an extended pre-incubation step.

The purpose of the second re-embedding gel is to anchor the sample gel to the glass by incorporating the acryloyl groups on the glass surface into the second re-embedding polyacrylamide gel, which penetrates into the sample gel. The re-embedding gel also supports the sample gel around the sides.

In addition, fluorescent beads are added to the gelling solution to help with the color-correction step in the computational processing of ExSeq data.

7.1 Preparation of Re-embedding Gelling Solution and Gelling Chamber

10m

Prepare re-embedding gelling solution as follows. Add reagents in the following order: Water, Acrylamide/Bis, Tris Base, 10% TEMED, 10% APS, TetraSpeck beads. Mix by vortexing after adding each chemical reagent. Do not vortex after adding TetraSpeck beads -- mix by pipetting up and down.

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A	B	C	D
Solution	**Stock Concentration**	**Volume**	**Final Concentration**
Acrylamide/Bis 19:1 solution	40% (w/v)	100 µL	~3.8% (w/v) acrylamide; 0.2% (w/v) N-N'-Methylenebisacrylamide
Water		875 µL	
Tris Base	1 M	5 µL	5 mM
10% TEMED	10% (w/v)	5 µL	0.05%
10% APS	10% (w/v)	5 µL	0.05%
0.2 µm TetraSpeck beads		10 µL	
Total		**1000 µL**	

Re-embedding gelling solution

Pipette a droplet of the re-embedding solution to a well of a Bind-Silane treated plate.

Transfer the sample gel to the well with a paintbrush and place the gel on top of the droplet of re-embedding solution. Orient the sample so that the tissue is on the top of the sample (farthest from the glass)*

Pipette another droplet of the re-embedding solution on top of the sample.

Place a 10 mm circular coverglass on top with tweezers (or larger coverglass if the sample is larger).

Using a pipette, backfill the remaining volume under the coverglass and around the gel.

*The sample orientation can be checked before the second re-embedding by staining with DAPI, imaging the sample, taking note of the in-focus Z-position, then flipping the gel and noting the in-focus Z-position again. The orientation with the higher in-focus Z-position is the orientation to use when gelling.

7.2 Nitrogen Purge and Second Re-embedding Gelation

1h 40m

Place 24-well plate into a humidified Tupperware container with two small holes in the lid and close the container.

Purge Tupperware container with nitrogen gas. To do this, start a slow flow of nitrogen gas into a nitrogen line connected to tubing with a blunt needle at the end. The flow rate should be barely perceivable when pointed at skin.

Insert the needle through one of the holes in the lid of the container. Ensure that the other hole is open, allowing for airflow out of the container. Ensure that the container does not immediately bulge or pop-open, which would be indicative of too high of a flow rate.

Purge for **00:10:00** .

After purging, withdraw the needle, and seal both holes by covering with tape.

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Cast re-embedding by incubating for **01:30:00** at **37 °C** .

7.3 Post-Gelation Wash

1h

Remove coverglass with tweezers.

Wash sample with 1X PBS for **00:20:00 x 3 times** at **Room temperature** .

--- Pause Point ---

The plate can be stored at **4 °C** for up to two weeks.

TdT treatment & Primer Hybridization 5h

8 Overview of Sequencing Preparation

4h 30m

In this step, we prepare the glass-anchored sample for *in situ* sequencing with the Illumina chemistry. There are two primary steps here: (1) capping terminal 3' DNA ends of cellular DNA with dideoxy nucleotides; and (2) hybridization of the Illumina sequencing primer.

9 Dideoxy Capping of 3' DNA Strands

2h 30m

To minimize background from base addition to exposed 3' ends of cellular DNA during the Illumina incorporation, Terminal deoxynucleotidyl transferase (TdT) is used to add dideoxynucleotides to block further extension.

9.1 Pre-incorporation

20m

Prepare TdT pre-incorporation mix and add to sample for **00:20:00** at **Room temperature** .

A	B	C	D
Reagent	**Stock Concentration**	**Volume**	**Final Concentration**
TdT buffer	10X	20 µL	1X
CoCl2	2.5 mM	20 µL	250 µM
ddNTP	2.5 mM each ddNTP	4 µL	50 µM each ddNTP
ddH2O		156 µL	
Total		**200 µL**	

TdT pre-incorporation mix

9.2 TdT Capping

1h 30m

Prepare TdT reaction mix, add to sample, and incubate for **01:30:00** at **37 °C** .

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A	B	C	D
Reagent	**Stock Concentration**	**Volume**	**Final Concentration**
TdT buffer	10X	20 µL	1X
CoCl2	2.5 mM	20 µL	250 µM
ddNTP	2.5 mM each ddNTP	4 µL	50 µM each ddNTP
TdT enzyme	20 U/µL	4 µL	0.4 U/µL
ddH2O		152 µL	
Total		**200 µL**	

TdT Reaction Mix

9.3 PBS Washes

30m

Wash gels with 1X PBS for **00:10:00 x 3 times** at **Room temperature** .

10 Sequencing Primer Hybridization

2h

In this step, the sequencing primer is hybridized to the amplicons. The sequence for the Illumina sequencing primer used for the padlock probes designed in the probe design protocol is TCT CGG GAA CGC TGA AGA CGG C, and should be ordered with HPLC purification (see materials).

10.1 Sequencing Primer Hybridization

1h

Prepare the sequencing primer hybridization mix by diluting the 100 µM sequencing primer stock by 1:40 in 4X SSC, forming a 2.5 µM sequencing primer mix.

Incubate gel with the sequencing primer hybridization mix in 4X SSC for **01:00:00** at **37 °C** .

10.2 SSC Washes

30m

Wash gels with 4X SSC for **00:10:00 x 3 times** at **37 °C** .

10.3 PR2 Washes

30m

Wash gel with PR2 buffer for **00:10:00 x 3 times** at **Room temperature** .

In Situ Illumina Sequencing: Overview 5h

11 Overview

The next three sections cover the process of in situ Illumina sequencing-by-synthesis.

The first step is elongation, the addition of a new base (Step 12). The second step is imaging (Step 13). The third step is cleavage (Step 14). These steps are repeated for each base of the barcode. For the barcode structure described by this protocol, a total of seven rounds of sequencing are required.

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Illumina Sequencing: Elongation 3h

12 Overview

1h 45m

In this step, the next base of the barcode is synthesized. This base is complementary to the template base of the barcode in the amplicon.

12.1 Incorporation Mix Pre-incubation

30m

Prepare ISS Incorporation Mix: 0.5X IMT and 2.5 mM MgCl2 in 1X Taq Reaction Buffer.

A	B	C	D
Reagent	**Stock Concentration**	**Volume**	**Final Concentration**
IMT (from Illumina kit)	1X	500 µL	0.5X
MgCl2	25 mM	100 µL	2.5 mM
Taq Reaction Buffer	10X	100 µL	1X
ddH2O		300 µL	
Total		**1000 µL**	

ISS Incorporation Mix

Wash sample with ISS Incorporation Mix for **00:15:00 x 2 times** at **Room temperature** .

12.2 Incorporation

10m

Treat sample with ISS Incorporation Mix for **00:10:00** at **50 °C** .

12.3 Post-incorporation Washes

45m

Wash with 2% Zwittergen (w/v) in PR2 for **00:15:00 x 2 times** at **50 °C** .

If harsher washing is needed, add 0.1 M MES buffer, pH 6.

Then, wash with PR2 for **00:15:00** at **50 °C** .

12.4 DAPI Staining

15m

Stain with DAPI (1 mg/L) in PR2 for **00:15:00** at **Room temperature** .

Illumina Sequencing: Imaging 15m

13 Overview

In this step, we transfer the sample to the Illumina imaging buffer and image the current round of the barcode.

13.1 Washing with Imaging Buffer

30m

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Wash samples with USM buffer (from Illumina kit) for **00:15:00 x 2 times** at **Room temperature** .

The sample is now ready for imaging. Keep the sample in USM buffer.

13.2 Overview of Imaging

The specifics of imaging vary significantly from microscope to microscope.

In general, users will be performing tiled array imaging, where each tile is a multicolor Z-section.

We recommend a 2% overlap between tiles to minimize photobleaching of amplicons at the edges of tiles. We recommend a spacing of 0.4 µm between adjacent Z-sections, and we recommend setting up the image acquisition to image all Z-places for a color channel before moving on to the next color channels. Imaging should be performed from longest to shortest excitation wavelength. Take care when setting up the image acquisition to ensure that the sample will be fully covered in Z across the entire XY region, since a small sample tilt can result in a significant fraction of the sample being out of the imaged volume.

This protocol is optimized for imaging on a Dragonfly spinning disk confocal microscope, using a Nikon 40X water immersion, long working distance, NA 1.15 objective (MRD77410). The key elements are shown in the following table. All lasers are set to 100% output power.

A	B	C	D	E
Channel Description	**Excitation (nm)**	**Excitation Laser Power (mW)**	**Emission Range (nm)**	Exposure time (ms)
Template Base G	685	30	705-845	400
Template Base T	640	160	663-737	200
Template Base A	561	150	575-590	200
Template Base C	488	150	500-550	400
DAPI	405	100	440-460	300

Summary of imaging parameters.

If a 685 laser is not available, the 640 laser can be used to excite template base G (added base C), and the signal can be separated from Template Base T (added base A) using a 775/140 emission filter (or similar). This will have slightly lower signal to background.

Illumina Sequencing: Cleavage 1h

1h 40m

14 Overview

In this step, the Illumina reversible terminator is unblocked, allowing for synthesis of the next base. The fluorophore added with the last base is also cleaved off the sequencing product.

20m

14.1 Post-Imaging Washes

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Wash with PR2 buffer for **00:10:00 x 2 times** at **Room temperature** .

14.2 Pre-incubation with Cleavage Buffer

20m

Wash with CMS (cleavage buffer from Illumina Kit) for **00:10:00 x 2 times** at **Room temperature** .

14.3 Cleavage

20m

Treat sample with CMS (cleavage buffer from Illumina Kit) for **00:20:00** at **50 °C** .

14.4 Post-Cleavage Washes

40m

Wash with PR2 buffer for **00:10:00 x 2 times** at **50 °C** .

Then, wash with PR2 buffer for **00:10:00 x 2 times** at **Room temperature** .

15 Iteration

If more bases need to be sequenced, **go to step #12** .

[OPTIONAL] Antibody Signal Recovery and Imaging 1h

16 Recovery of Antibody Staining

1d

If antibody staining with a biotinylated secondary antibody was previously performed in the tissue processing step, this section recovers and images that signal.

For imaging the antibody staining: if sub-voxel resolution registration to the in situ sequencing data will be performed, skip the final cleavage step after the last base of sequencing and image the antibody staining signal with the last base of sequencing. The puncta signal can be used in the computational processing to co-register this dataset.

If another feature (i.e. DAPI) will be used to coregister the antibody staining, the cleavage can be performed before antibody staining signal recovery.

16.1 [Only if Final Cleavage is Skipped] PR2 Wash

30m

Wash the samples with PR2 buffer for **00:30:00** at **Room temperature** .

16.2 Stain with Fluorophore-Labeled Streptavidin

1d

Incubate the samples with 10 µg/mL fluorophore-labeled Streptavidin (i.e. Streptavidin-Alexa 488) in PR2 for **Overnight** at **4 °C** .

The next day, wash samples with PR2 for **00:30:00 x 3 times** at **Room temperature** .

16.3 DAPI Staining

15m

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Stain with DAPI (1 mg/L) in PR2 for **00:15:00** at **Room temperature** .

16.4 Washing with Imaging Buffer

15m

Wash samples with USM buffer (from Illumina kit) for **00:15:00 x 2 times** at **Room temperature** .

Add USM buffer to the sample again.

16.5 Imaging

The precise imaging parameters depend on the specifics, but the imaging protocol for sequencing may serve as a template.

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