Oct 01, 2024 Version 2

AAV-Zombie on cultured cells V.2

DOI
dx.doi.org/10.17504/protocols.io.36wgqnz53gk5/v2

Gerard Michael Coughlin[1]

[1]California Institute of Technology

Gerard Michael Coughlin
California Institute of Technology

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.36wgqnz53gk5/v2

Protocol Citation: Gerard Michael Coughlin 2024. AAV-Zombie on cultured cells. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.36wgqnz53gk5/v2**Version created by **Gerard Michael Coughlin**

Protocol status: Working
We use this protocol and it's working

Created: September 17, 2024

Last Modified: October 01, 2024

Protocol Integer ID: 108761

Keywords: Spatial genomics, adeno-associated viruses, in situ detection

Funders Acknowledgement:
Aligning Science Across Parkinson's
Grant ID: ASAP-020495

Abstract

Detection of AAV genomes *in situ* can facilitate understanding of AAV transduction and processing. This protocol enables detection of AAV genomes and concatemers in cultured cells and is based on the Zombie method published in Askary et al., *Nat Biotechnol 38*: 66. AAVs carrying barcoded genomes are delivered to cells. Following fixation, a phage RNA polymerase is used to transcribe the barcode *in situ,* yielding barcoded transcripts that can be detected with RNA FISH. This protocol is compatible with either ssAAV or scAAV genomes. In the former case, detected genomes will be primarily nuclear and detectable once second strand synthesis has occurred. In the latter case, the genome can be detected regardless of where in the transduction pathway the genome is. This protocol can be combined with RNA FISH detection of reporter transcripts or endogenous transcripts, enabling measurement of expression and cell type classification.

Materials

- Methanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**322415
- Acetic Acid 100% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A6283
- Ethanol, anhydrous **VWR International Catalog #**89125-172
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023
- Paraformaldehyde 32% (methanol free) **Electron Microscopy Sciences Catalog #**15714
- PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific Catalog #**AM9625
- SSC (20X), RNase-free **Thermo Fisher Catalog #**AM9763
- Ethylene carbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E26258
- Dextran Sulfate, 50% solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**3730-OP
- Tween 20, 10% aqueous solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**11332465001
- MEGAscript™ T7 Transcription Kit **Thermo Fisher Scientific Catalog #**AMB13345
- MEGAscript™ SP6 Transcription Kit **Thermo Fisher Scientific Catalog #**AM1330
- Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution in Water **Thermo Fisher Scientific Catalog #**H3570
- ProLong Diamond Antifade Mountant **Thermo Fisher Scientific Catalog #**P36970

HCR FISH probes - can be obtained as ssDNA oligos, from Integrated DNA Technologies (IDT; **https://www.idtdna.com/**) and/or from Molecular Instruments (**https://www.molecularinstruments.com/**)
HCR hairpins - can be obtained from Molecular Instruments (**https://www.molecularinstruments.com/**)
Fluorophore-conjugated polyT probes can be obtained from Integrated DNA Technologies (IDT; **https://www.idtdna.com/**)

For paired IHC and AAV-Zombie, you will also need:

- BlockAid™ Blocking Solution **Thermo Fisher Scientific Catalog #**B10710
- Triton X-100, 10% solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**93443

Suitable primary and secondary antibodies (e.g.

- anti-AAV VP1/VP2/VP3 mouse monoclonal, B1, AFDye™ 488 Conjugate **PROGEN Catalog #**61058-488)

Note

We have successfully detected capsid proteins with immunofluorescence staining via a monoclonal antibody against linear capsid epitopes (clone B1). As MAA fixation is expected to denature proteins, we suggest trying primary antibodies against linear epitopes vs. those against conformational epitopes.

Safety warnings

- AAVs are biohazardous materials and must be handled according to governmental and institutional regulations. Experiments involving AAVs were performed using biosafety level 2 practices as required by the California Institute of Technology and the US Centers for Disease Control and Prevention.
- rAAVs, although replication-incompetent, are potent gene-delivery vehicles and must be handled according to governmental and institutional regulations. The safety of packaged transgenes (e.g., oncogenic genes) should be carefully considered. Perform all procedures in a certified biosafety cabinet and clean AAV-contaminated equipment, surfaces, and labware with fresh 10% (vol/vol) bleach.
- HEK293T cells and AAVs are biohazardous materials and must be handled according to governmental and institutional regulations. All experiments involving the aforementioned materials were performed in a Class II biosafety cabinet with annual certification as required by the California Institute of Technology and the US Centers for Disease Control and Prevention.

Before start

This protocol enables spatial detection of AAV genomes and concatemers in cultured cells. For detection of AAV genomes, a Zombie barcode and phage RNA polymerase promoter are required in a single AAV genome. For detection of AAV concatemers (SpECTr), 2 AAV genomes are required: one genome carries a Zombie barcode and the other carries a phage RNA polymerase promoter. Barcode sequences can be found in Supplementary Table 1 of Askary et al., *Nat Biotechnol 38*: 66.

See https://www.biorxiv.org/content/10.1101/2023.12.23.573214v1 for more information on these methods.

Reagent set up

1 General note on reagents and consumables

> **Note**
>
> As AAV-Zombie detects AAV genomes through an RNA transcript proxy, it's key that all reagents, tools, and work surface are RNase-free. Use RNase-free consumables and reagents to prepare all buffers.

2 Prepare 3:1 methanol:acetic acid (MAA) fixation buffer by combining:

- Methanol: 30 mL
- Glacial acetic acid: 10 mL

in a 50 mL conical tube. Store solution at -20 °C for up to 3 months.

> **Note**
>
> Scale up or down volumes based on needs.

3 Prepare 70% ethanol solution, by combining

- 100% Ethanol: 35 mL
- UltraPure water: 15 mL

in a 50 mL conical tube. Store solution at -20 °C for up to 3 months.

> **Note**
>
> Scale up or down volumes based on needs.

4 Prepare 4% paraformaldehyde, 1x PBS solution (4% PFA, 1x PBS), by combining:

- 32% paraformaldehyde: 6.25 mL
- 10x Phosphate-buffered saline (PBS): 5 mL
- UltraPure water: 38.75 mL

in a 50 mL conical tube. Store solution at 4 °C for up to 1 month.

5 Prepare probe hybridization buffer, by combining:

- 20x saline-sodium citrate (SSC) buffer: 5 mL
- Ethylene carbonate: 5 mL
- 50% dextran sulfate: 10 mL
- UltraPure water: 30 mL

in a 50 mL conical tube. Store solution at Room temperature for up to 3 weeks, or -20 °C for up to a year.

Note

Scale up or down volumes based on needs. We make appropriately sized aliquots (e.g. 10 mL) and store at -20 °C .

Ethylene carbonate is a non-toxic substitute for formamide, and may also help to reduce background from non-specific interaction (as compared to formamide-based hybridization buffers). Substituting ethylene carbonate for formamide, at the same concentration, should yield qualitatively similar results.

Ethylene carbonate is solid at room temperature. Pre-warm to 37 °C to melt.

Probe hybridization buffer can also be obtained from Molecular Instruments (**https://www.molecularinstruments.com/**)

6 Prepare stringent wash buffer, by combining:

- 20x saline-sodium citrate (SSC) buffer: 5 mL
- Ethylene carbonate: 15 mL
- UltraPure water: 30 mL

in a 50 mL conical tube. Store solution at Room temperature for up to 3 weeks, or -20 °C for up to a year.

Note

Scale up or down volumes based on needs. We make appropriately sized aliquots (e.g. 10 mL) and store at -20 °C .

Ethylene carbonate is a non-toxic substitute for formamide, and may also help to reduce background from non-specific interaction (as compared to formamide-based wash buffers). Substituting ethylene carbonate for formamide, at the same concentration, should yield qualitatively similar results.

Stringent wash buffer can also be obtained from Molecular Instruments (as "Probe wash buffer"; **https://www.molecularinstruments.com/**)

7 Prepare wash buffer (5x SSC, with 0.1% Tween-20), by combining:

- 20x saline-sodium citrate (SSC) buffer: 100 mL
- 10% Tween-20: 5 mL
- UltraPure water: 395 mL

Store solution at Room temperature for up to a year.

8 Prepare 5x SSC, by combining:

- 20x saline-sodium citrate (SSC) buffer: 12.5 mL
- UltraPure water: 37.5 mL

Store solution at Room temperature for up to a year

9 Prepare HCR amplification buffer, by combining:

- 20x saline-sodium citrate (SSC) buffer: 5 mL
- Ethylene carbonate: 5 mL
- UltraPure water: 35 mL

in a 50 mL conical tube. Store solution at Room temperature for up to 3 months.

Note

Scale up or down volumes based on needs.

Ethylene carbonate is a non-toxic substitute for formamide, and may also help to reduce background from non-specific interaction (as compared to formamide-based wash buffers). Substituting ethylene carbonate for formamide, at the same concentration, should yield qualitatively similar results.

HCR amplification buffer can also be obtained from Molecular Instruments (**https://www.molecularinstruments.com/**)

10 Prepare 1x PBS, by adding:

- 10x PBS: 55.5 mL

To a 500 mL bottle of UltraPure water.

11 (Optional) Prepare 1x PBS-Tx, by combining:

- 10x PBS: 50 mL
- 10% Triton X-100: 5 mL
- UltraPure water: 445 mL

Note

1x PBS-Tx is used if you opt to add incorporate antibody labeling to the protocol.

Sample preparation

12 General note on cell culture.

Note

The volumes for this protocol assume that the samples are adherent cultured cells plated on a 12 mm coverglass in a 24-well plate. All volumes in this section are per well. Scale volumes up and down accordingly if using different sized coverslips, or if cells cultured on a glass-bottom dish.

If cells are cultured and processed on the well bottom, use glass-bottom well to ensure optimal conditions for imaging.

Note that given the cost of the reagents involved in AAV-Zombie, scaling up reactions can become costly. Flooding wells with RNA phage polymerase solution requires at least 10-times more buffer and enzyme than if the reaction is carried out by overturning a coverslip on a droplet of solution. Thus, we recommend culturing cells on coverslips, or passaging cells onto coverslips before fixation.

Ensure that coverslips used have adequate and appropriate coatings for cell type of interest.

13 Transduce cells with AAVs carrying Zombie barcode and phage RNA polymerase promoter (barcode sequences can be found in Supplementary Table 1 of Askary et al., *Nat Biotechnol 38*: 66).

Note

This protocol enables spatial detection of both AAV genomes and concatemers in cultured cells. For detection of AAV genomes, a Zombie barcode and phage RNA polymerase promoter are required in a single AAV genome. For detection of AAV concatemers (SpECTr), 2 AAV genomes are required: one genome carries a Zombie barcode and the other carries a phage RNA polymerase promoter. Barcode sequences can be found in Supplementary Table 1 of Askary et al., *Nat Biotechnol 38*: 66.

Fixation time(s), cell type, AAV serotype, genome form, MOI, etc. are all experiment-dependent. We have used this protocol to detect scAAV and ssAAV genomes delivered by AAV6, AAV-DJ, and engineered AAV9 variants.

In choosing a phage RNA polymerase and promoter, we have relied primarily on T7. We have also used SP6 RNA polymerase successfully, but this yields smaller size spots. We have not tested T3 RNA polymerase in this application, but according to Askary et al., *Nat Biotechnol 38*: 66, this should yield similar results to T7 RNA polymerase.

14 (Optional) Depending on cell type, experimental question, AAV genome format, etc., it may be necessary to change the media some time post-transduction (e.g. >3 hr). Between aspiration of the old media and addition of the new media, we recommend doing at least 3 washes in a suitable buffer or media.

Note

Given their double stranded nature, scAAV genomes will be detected by AAV-Zombie regardless of where they are in the transduction pathway. This includes AAVs that are merely adsorbed to the surface of the culture dish or coverslip. To reduce background from scAAVs outside of cells, we recommend thoroughly washing cells after transduction.

15 (Optional) Dense adherent cultures can complicate cell segmentation and downstream image analysis. It may be beneficial to passage cells to a lower confluence (e.g. 10%) before fixation.

Note

The time that it takes for cells to reattach after passaging is based on multiple factors (cell type, passaging method, media, coatings, etc.). Visually confirm that cells are attached before proceeding with fixation.

16 At time of collection, aspirate media and wash cells one time with 1 mL DPBS. Aspirate DPBS and add 1 mL ice-cold MAA fixation buffer. Transfer to -20 °C for 00:15:00 . 15m

Note

Collection time is dependent upon experimental question. Because AAV-Zombie detects the AAV's genome, there is no reason to wait until reporter gene expression (e.g. GFP). However, as AAV-Zombie is compatible with RNA-based readouts of reporter gene expression, it may be beneficial to wait until detectable reporter expression to allow for paired DNA and RNA readout.

MAA fixes cells by causing proteins to precipitate, but will not crosslink molecules. Avoiding crosslinking fixatives is necessary as crosslinking will interfere with the *in situ* transcription reaction.

17 Aspirate MAA into an appropriate container for disposal. Wash cells in 1 time with 1 mL ice-cold 70% ethanol. Remove ethanol wash and dispose of with MAA. Add 1 mL of ice-cold 70% ethanol and transfer samples to -20 °C until processing.

Note

We have processed samples that were stored up to 3 months in 70% ethanol at -20 °C, and achieved the expected results.

In situ transcription and probe hybridization

1h

18 General note on scaling reactions

Note

Note that given the cost of the reagents involved, scaling up reactions can become costly; flooding wells with the phage RNA polymerase solution requires at least 10-times more buffer and enzyme than if reaction is carried out by overturning a coverslip on a droplet of the phage RNA polymerase solution. Thus, we recommend culturing cells on coverslips, or passaging cells onto coverslips before fixation.

All volumes and procedures in following section assume that the samples are adherent cultured cells on a 12 mm coverslip.

19 Prepare the reaction chamber. Remove the wafer from a P1000 pipette tip box, add 50 mL of MilliPore (or comparable) water to the bottom, and then replace the wafer. Cut an appropriately sized piece of parafilm and lay it over the top of the wafer with the clean side up (i.e. the side that was facing the paper). 1h

Place the box into a 37 °C incubator, for at least 01:00:00 .

Note

This pipette tip box will serve as a humidified reaction chamber for the *in situ* transcription reaction to take place. The reaction solution will be pipetted into droplets onto the surface of the parafilm, and thus it is critical that the upwards-facing side remains clean. Prewarming the parafilm will allow it to flatten out.

20 Thaw the NTP(s) and buffer at Room temperature, then place on ice.

Note

The suggested kit provides each nucleoside triphosphate (NTP) separately (i.e. ATP, UTP, CTP, GTP). To simplify reaction setup, mix together NTPs at equal volumes, then aliquot and store at -20 °C .

21 Set up *in situ* transcription reaction solution. For each coverslip, prepare 20 µL of reaction solution, consisting of:

- NTPs: 8 µL (or 2 µL of each NTP, if storing separately)
- UltraPure water: 8 µL
- RNA polymerase: 2 µL
- Buffer: 2 µL

Store reaction solution on ice until use.

Note

The recommended T7 and SP6 RNA polymerase *in vitro* transcription kits use separate buffers and separate NTP concentrations. Use the buffers and NTPs specific to the polymerase used. If combining T7 and SP6 polymerases in a single reaction, use T7 buffer and T7 NTPs.

Prepare extra transcription solution than necessary, especially if processing many coverslips.

22 Retrieve samples from -20 °C storage. Aspirate the 70% ethanol off, briefly wash samples with 1 mL of 1x PBS. Aspirate wash, add 1 mL of fresh 1x PBS and keep at Room temperature until use.

23 Set up tools and disposables. You will need a Kimwipe, a pair of clean forceps (suitable for lifting and manipulating coverslips), a 23g needle with the tip bent (to facilitate lifting of coverslip from the well), and a 15 mL tube with 15 mL of UltraPure water.

24 Start *in situ* transcription reactions.

CRITICAL For these steps, pay careful attention to which side of the coverslip has the cells.

1. Remove reaction chamber from 37 °C incubator. Pipette 20 µL of *in situ* transcription solution onto the surface of the parafilm (if doing multiple coverslips, prepare up to 4 spots at a time; ensure spots are adequately spaced)
2. Gently lift coverslip from well, using hooked needle and forceps.
3. Briefly dip coverslip in UltraPure water to remove residual PBS, then gently touch the edge of the coverslip to a Kimwipe to wick away excess liquid
4. Carefully lower coverslip with cell-side down onto a droplet of the transcription solution, being careful not to trap bubbles underneath
5. Repeat for each coverslip

25 Transfer reaction chamber to 37 °C incubator, and incubate transcription reaction for 2-3 hours.

Note

During this incubation, the phage RNA polymerase transcribes the barcode on the AAV genome, resulting in free-floating RNA transcripts containing the barcode sequence.

26 During *in situ* transcription reaction, pre-warm the hybridization buffer to 42 °C . You will need 1 mL of hybridization buffer per coverslip.

Note

500 µL of the hybridization buffer will be used in a pre-hybridization equilibration step (Step 26) and 500 µL will be used for probe hybridization (Step 28). Prepare extra hybridization solution.

27 Following *in situ* transcription reaction, use forceps to lift coverslips off parafilm and place into a clean 24-well plate, with the cell-side up. Add 500 µL of 4% PFA, 1x PBS solution. Fix cells for 00:15:00 at Room temperature . Following fixation, aspirate PFA solution, then wash twice with 1 mL of 1x PBS, followed by 2 washes in 5x SSC (00:05:00 per wash). 20m

Note

PFA will crosslink molecules in the samples, including nascent RNA from the *in situ* transcription reaction. This fixation step ensures that barcoded transcripts do not diffuse from the template DNA, enabling spatial detection of the AAV genome through the RNA proxy.

28 After PFA fixation, remove 5x SSC and add 500 µL of pre-warmed hybridization buffer. Incubate at 37 °C for 00:30:00 . 30m

29 During incubation in Step 26, prepare probe solution by diluting probes to 2 nM in the remaining hybridization buffer. Return to 42 °C until use.

Note

Probes for Zombie barcodes, AAV reporter gene transcripts and for endogenous transcripts can be pooled at this point.

Probes can be obtained as ssDNA oligos, e.g. from Integrated DNA Technologies (IDT).

We store working solutions of probes at 1 uM in -20 °C . These are prepared either by combining and diluting 100 uM stocks of each separate probe, or by obtaining a pre-pooled mixture of probes from IDT and resuspending these to 1 uM.

30 Following equilibration in hybridization buffer, aspirate old hybridization buffer from samples and add 500 µL of probe solution (Step 27). Wrap seal edge of the plate with parafilm to prevent evaporation. Incubate at 37 °C Overnight .

Probe washes and Hybridization chain reaction (HCR) amplification 30m

31 Note on reaction volumes

Note

All volumes and procedures in following section assume that the samples are adherent cultured cells on a 12 mm coverslip.

32 Pre-warm stringent wash buffer to 37 °C, vortexing a couple times to ensure components are thoroughly mixed. You will need 1.5 mL of stringent wash buffer per coverslip.

33 Once stringent wash buffer is warmed to 37 °C and mixed, wash samples 3 times in stringent wash buffer. Perform 1 brief rinse with 500 µL of buffer, followed by two longer washes with 1 mL at 37 °C for 00:30:00 each. 30m

> **Note**
>
> Keep stringent wash buffer at 37 °C during washes.

34 Following stringent washes, wash 3 times in wash buffer (5x SSC, with 0.1% Tween-20). Perform 1 brief rinse with 1 mL of buffer, followed by two longer washes with 1 mL at Room temperature for 00:30:00 each. 30m

35 Following washes, aspirate wash buffer from samples and add 1 mL of HCR amplification buffer. Equilibrate samples in HCR amplification buffer for 00:30:00 at Room temperature. 30m

36 During HCR amplification buffer equilibration step, prepare HCR hairpins. 12m

1. Aliquot each hairpin separately into PCR tubes
2. Using a thermocycler, heat hairpins to 95 °C for 00:02:00
3. Remove hairpins from thermocycler and allow to cool back down to room temperature in the dark (at least 00:10:00

> **Note**
>
> Snap-cooling hairpins is critical, as it allows the hairpins to re-fold properly.
>
> **CRITICAL** Do not mix hairpins together before snap-cooling, as this may cause them to hybridize to one another. Only mix the hairpins together in amplification buffer, and only once they have cooled down to room temperature.

37 Following equilibration in HCR amplification buffer, prepare HCR amplification solution. For each coverslip, prepare 300 µL of amplification solution. Dilute snap-cooled hairpins to 30 nM in the HCR amplification buffer.

For example, for one coverslip with one probe set using the B5 initiator, combine:

- HCR amplification buffer: 294 µL
- Hairpin 1 (e.g. B5H1): 3 µL
- Hairpin 2 (e.g. B5H2): 3 µL

Briefly vortex to mix.

Note

Prepare extra amplification solution.

38 Aspirate old amplification buffer from samples, then add 300 µL of amplification solution. Incubate 01:00:00 to 03:00:00 at Room temperature and in the dark. 4h

Note

From this point on, keep samples in dark as much as possible.

A 1 hour incubation may be preferred, to limit the size of resulting spots. However, if also detecting endogenous genes or reporter transcript, it may be necessary to do a 3 hour incubation. Alternatively, incubate samples in amplification solution with hairpins for endogenous genes for 2 hours, then switch to an amplification solution containing all hairpins for the final hour.

39 Following HCR amplification, remove unassembled hairpins by washing 4 times in wash buffer (5x SSC, with 0.1% Tween-20). Perform 1 brief rinse with 1 mL of buffer, followed by three longer washes with 1 mL at Room temperature for 00:10:00 each. 10m

40 (Optional) Label cell cytoplasm with a fluorophore-conjugated polyT probe. For each coverslip, prepare 1 mL of a 100 nM polyT probe dilution in wash buffer (5x SSC, with 0.1% Tween-20). Incubate samples in polyT probe for 01:00:00 at Room temperature . 1h 10m

Following incubation, wash samples 4 times in wash buffer (5x SSC, with 0.1% Tween-20). Perform 1 brief rinse with 1 mL of buffer, followed by three longer washes with 1 mL at Room temperature for 00:10:00 each.

41 (Optional) Label proteins with immunofluorescence staining. 1h 15m

We expect that most standard immunofluorescence protocols can be used here. Below is the protocol we used for labeling AAV capsid proteins with immunofluorescence using a fluorophore-conjugated primary antibody. Optimal incubation conditions, dilutions, and durations, will need to be determined empirically for each protocol and antibody.

1. Block samples in 1 mL of BlockAid blocking buffer with 0.1% Triton X-100, for 01:00:00 at Room temperature
2. Incubate samples with 1:100 dilution of primary antibody in BlockAid blocking buffer with 0.1% Triton X-100, 4 °C for Overnight .
3. Wash samples 3 times in 1x PBS-Tx, for 00:15:00 each at Room temperature

Note

We successfully detected capsid proteins with immunofluorescence staining via a fluorophore-conjugated monoclonal antibody against linear capsid epitopes (clone B1). As MAA fixation is expected to denature proteins, we suggest trying antibodies against linear epitopes vs. those against conformational epitopes.

42 (Optional) Label nucleus with Hoescht 33342. For each coverslip, prepare 1 mL of 1x PBS with 1/10000 Hoechst 33342. Incubate samples in Hoechst solution for 00:15:00 at Room temperature . 20m

Following incubation, wash samples 3 times with 1 mL of 1x PBS at Room temperature for 00:05:00 each.

Note

DAPI can be used in place of Hoechst, with no changes to the protocol.

43 Mount coverslips. You will need a Kimwipe, a pair of clean forceps (suitable for lifting and manipulating coverslips), a 23g needle with the tip bent (to facilitate lifting of coverslip from the well), and a 15 mL tube with 15 mL of UltraPure water, a suitable mounting medium, and glass slides.

For each coverslip:

1. Place a 20 µL droplet of mounting medium on the surface of a clean glass slide.
2. Using a hooked needle and forceps, lift coverslip out of the well, dip briefly into UltraPure water, then touch side of coverslip to a Kimwipe to wick away excess water.
3. Carefully lower coverslip cell side-down onto the droplet of mounting medium, being careful to prevent bubbles

Once all coverslips are mounted, gently press down on each coverslip to push out excess mounting media. Store in the dark at Room temperature for at least Overnight until mounting media cures. Once cured, excess Prolong Diamond can be easily removed by running slides under a gentle stream of Millipore water, being careful to not displace coverslips.

Samples are now ready to image.

Note

For mounting coverslips, we use Prolong Diamond, but any mounting medium compatible with fluorescence signal should work. If using another mounting medium, follow manufacturer directions for that mounting medium.

Protocol references

Askary, A. *et al.* In situ readout of DNA barcodes and single base edits facilitated by in vitro transcription. *Nat. Biotechnol.* **38**, 66–75 (2020).

Jang, M. J. *et al.* Spatial transcriptomics for profiling the tropism of viral vectors in tissues. *Nat. Biotechnol.* **41**, 1272–1286 (2023).

Choi, H. M. T. *et al.* Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *Development* **145**, dev165753 (2018).