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Controls, imaging, and analysis of AAV-Zombie and SpECTr experiments

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Gerard Michael Coughlin[1]

[1]California Institute of Technology

Gerard Michael Coughlin
California Institute of Technology

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We use this protocol and it's working

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Abstract

This protocol describes best practices for designing, imaging, and analyzing AAV-Zombie and SpECTr experiments.

For more information on these methods, see:

https://www.biorxiv.org/content/10.1101/2023.12.23.573214v1
dx.doi.org/10.17504/protocols.io.14egn6k7yl5d/v1
dx.doi.org/10.17504/protocols.io.36wgqnz53gk5/v2

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.

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.

Ethics statement

Animal husbandry and all procedures involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee (IACUC) and by the Office of Laboratory Animal Resources at the California Institute of Technology.

Controls for AAV-Zombie and SpECTr experiments

1 This section outlines best practices for designing AAV-Zombie and SpECTr experiments. We divide these into 'necessary' and 'recommended' controls.

We strongly recommend including 'necessary' controls in every AAV-Zombie experiment, and these should be performed in the tissue and/or cell types of interest, and processed along with experimental samples.

'Recommended' controls may be helpful in the first iteration of the experiment or when learning the methods, but are not always necessary for clear interpretation of the data.

2 **Necessary controls**

In addition to hybridizing to the target *in situ* transcribed barcode, HCR probes may also hybridize to single-stranded AAV genomes or to barcoded transcripts originating from transcription by promoter elements in the AAV ITR.

- For SpECTr experiments, a **barcode-only control**, in which cells of interest are transduced with only the barcode AAV and not the T7/SP6 AAV, can help to distinguish 'real' and 'artifactual' signal
- Alternatively, and for AAV-Zombie experiments detecting a single AAV genome (not concatemer), a **'no phage RNA-polymerase' control** can be used (i.e. leave out the T7, SP6, or T3 phage RNA polymerase from the *in situ* transcription mix for one coverslip or slide).

HCR can produce spots due to non-specific binding with other nucleic acids in the cells

- To ensure that imaging and analysis parameters are set to exclude such spots, run a **non-transduced or non-transfected control** in which the target sequence is not present

3 **Recommended controls**

For SpECTr experiments, running T7/SP6 only control, in which cells of interest are transduced with only the T7/SP6 AAV and not the barcode AAV, can help to convince that the SpECTr signal originates from AAV concatemers

Cells transfected with the pAAV plasmid containing the same phage RNA polymerase promoter and barcode as experimental conditions can serve as a positive control, to ensure that AAV-Zombie readout was successful.

4 **Other considerations**

In addition to controls, consider what other markers are necessary for the experimental question and planned downstream analysis. E.g. Hoechst or DAPI, a marker for the cell body, etc.

Imaging of AAV-Zombie and SpECTr experiments

5 This sections outlines best practices for imaging of AAV-Zombie and SpECTr experiments.

Microscope choice

In general, a microscope that is suitable for imaging single-molecule FISH experiments should be suitable for AAV-Zombie and SpECTr experiments. For example, laser scanning or spinning disk confocal should be adequate. An epifluorescence microscope with a cooled CCD camera may also work. A high magnification lens (40x or greater), with a high NA is preferred.

Image acquisition

When setting imaging parameters, aim to capture full dynamic range of the signal without saturating pixels. If possible, adjust laser power before adjusting gain. Use control samples (e.g. barcode-only control, non-transduced control) to check that non-specific signal is distinguishable from real signal at the set imaging parameters.

Image control samples at the same parameters as experimental samples. These can be useful when analyzing data, if necessary to further distinguish real from artifactual spots.

To avoid bias when choosing fields of view (FOVs) on experimental samples, set FOVs while imaging a non-experimental channel (e.g. Hoechst).

Analysis of AAV-Zombie and SpECTr experiments

6 Multiple image analysis tools are available and can help with analysis of AAV-Zombie and SpECTr experiments:

- For automated segmentation of cells and nuclei from cultured cells and from tissue, we used Cellpose and napari
- For manual segmentation of cells and tissues we used the Fiji distribution of ImageJ
- For object detection and measurement, and for measurement of fluorescent protein or FISH signal intensity, we used CellProfiler

When analyzing these data, use control samples to set thresholds to ensure that you are effectively filtering out artifactual signal.

Protocol references

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