# inoFISH: inosine fluorescence in situ hybridization for visualizing adenosine to inosine RNA editing in single mammalian cells

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# Abstract

inoFISH is a fluorescence _in situ_ hybridization-based method for visualizing adenosine to inosine editing of bases at a known position in a transcript of interest in single mammalian cells. In preparation for inoFISH, one designs probe sets to the target transcript and to the target editing site based on the full transcript sequence and the position of the editing site. To perform inoFISH, samples are fixed and permeablized, then the guide probe and the detection probes are simultaneously hybridized to target transcripts in the cells. After washes, the samples are ready to image on a fluorescence microscope. Lastly, one calls guide and detection probe spots in the collected images and performs colocalization analysis to identify unedited and edited transcripts of interest in the observed samples. 10%-50% of transcripts will be so labeled, depending on the probe set. Counterstaining of cellular compartments may be combined with inoFISH to characterize subcellular localization of edited transcripts.


# Introduction

Please note that much of this protocol is adapted from Arjun Raj's RNA FISH protocol database: https://sites.google.com/site/singlemoleculernafish/home , accessed on April 30, 2017. Some text has also been copied for clarity from the main text of the Brief Communication \(Mellis et al. , Nat. Methods, 2017) with which this Protocol is associated. Adenosine to inosine RNA editing is a frequently observed type of RNA editing. Here we present inoFISH, a method for visualizing adenosine to inosine editing at a defined site in a transcript of interest. Discriminating edited from unedited RNA via RNA fluorescence in situ hybridization \(RNA FISH) is difficult because it relies on the hybridization of oligonucleotide probes to visualize the target of interest. Short oligonucleotides bind nonspecifically while long oligonucleotides cannot discriminate single-base differences. We thus use a 'toehold probe' strategy to reduce the initial hybridization region of our detection probes in order to confer selectivity based on single-nucleotide differences in the target RNA. Our scheme takes advantage of the fact that inosine preferentially binds to cytosine rather than thymine by using two detection probes that compete to target the unedited, adenosine-bearing sequence using a thymine, and the edited, inosine-bearing sequence using a cytosine. Upon specific binding, the "mask" sequence is released by strand displacement to stabilize hybridization. However, single oligonucleotides are still prone to nonspecific binding, so we simultaneously use smFISH \(the "mRNA guide" probe) to target a constant region of mRNA, coupled to a unique fluorophore . The mRNA guide shows us where to look for specific detection probes. The workflow for inoFISH is as follows: 1. Select a target editing site in a transcript of interest 2. Use bulk assay techniques, such as gDNA PCR and RT-PCR with Sanger sequencing, to preliminarily check for any editing at this site in biological samples of interest 3. Ensure that the target site is compatible with inoFISH probe design 4. Design a small panel of candidate inoFISH probe sets 5. Screen candidate inoFISH probe sets for optimal signal-to-noise ratio a. Computational calling of spots with manual curation of spot intensity thresholds b. This analysis pipeline is particularly time-consuming for detection probe spots, which require more frequent inspection than normal smFISH spots to distinguish them from background autofluorescence 6. Conduct inoFISH experiments a. Computational calling of spots with manual curation of spot intensity

thresholds 7. Editing level estimation and downstream analyses Please note that inoFISH experiments are time-consuming, due to the both the time required to experimentally optimize and validate a probe set and the time required to perform manual quality controls of detection probe spot calls. We strongly recommend familiarizing oneself with the basic smFISH protocol and analysis pipeline using rajlabimagetools prior to starting an inoFISH experiment.

# Reagents

Cell culture supplies ● Lab-Tek 2-well chambered coverglasses, Fisher, 12-565-471 ● Lab-Tek 8-well chambered coverglasses, Fisher, 12-565-470 Reagents for fixation, wash, hybridization, anti-fade ● NF H2O, Ambion 4387936 ● Formamide, Ambion AM9342 \(Aliquot and store at 4C; bring to room temperature before making buffers. Some people say this stuff goes bad really rapidly, but we haven't had a lot of trouble with that.) ● 20xSSC, Ambion AM9765 ● Dextran Sulfate, Sigma D8906-50G \(store at 4C) ● 10xPBS, Ambion AM9624 ● Glucose oxidase, Sigma G2133-10KU \(Dissolve 37mg in 10mL of 50mM Sodium Acetate, pH ~5.5 \(diluted from 3M, pH 5.5) for a 3.7mg/mL stock. Make a bunch of aliquots and store at -20C. Each aliquot is good for ~20+ freeze/thaws.) ● Catalase, Sigma C3515-10MG ● Tris pH 8, Ambion AM9856 ● 10% glucose solution \(wherever) \(For ethanol precipitation if you are coupling probes manually) ● 3M Sodium Acetate, pH 5.5, Ambion AM9740 Working solutions ● Fixation solution: ● 5mL 10x PBS ● 5mL 37% formaldehyde \(100% formalin) ● 40mL NF H 2O \(can leave in hood at RT for months). ● Wash buffer: ● 5mL 20x SSC ● 5mL formamide ● 40mL NF H 2O \(can leave in the hood at RT for months). ● 70% EtOH: ● 35mL 95% EtOH ● 15mL NF H 2O \(lasts forever). ● Hybridization buffer: 3 ● Add 1g dextran sulfate to 7mL NF H 2O and mix by rotation for a while \(could take several minutes to a half an hour). Then add 1mL formamide and 1mL 20x SSC and volume up to 10mL \(volume by eye in a 15mL Falcon tube is fine). Store at -20C in 500µL aliquots; good for years. ● Anti-fade buffer: ● Note on catalase: vortex thoroughly before use. Try not to keep the bottle open very long because it is sometimes prone to contamination. ● For the antifade buffer, use 850µl NF H 2O , 100µl 20x SSC, 40µl 10% glucose, 10µl Tris. Use 900µl of this for a pre-wash before adding the actual antifade solution itself. To the remaining 100µl, add 1µl each of glucose oxidase and catalase; this is the antifade solution for use with Cy5/Quasar 670. We use the following dyes for inoFISH: ● Guide probe: Cal Fluor 610 \(Stellaris probes, Biosearch Technologies) ● Detection probes: Cy3 and Cy5 ● Counterstain RNA FISH fluorophores: Atto 700, sometimes Atto 488 if your samples have very low autofluorescence Notes: It can be fairly tricky to separate Cy3 and Cal Fluor 610/Alexa 594. To do this, we use the narrow band Cy3 filter set \(SP102v1) and the custom filter set from Omega for Cal Fluor 610/Alexa 594. These filters could probably be optimized further, as well. Materials for bulk assays of RNA editing ● miRNeasy Mini Kit, Qiagen, 217004 ● DNeasy Blood & Tissue Kit, Qiagen, 69504 ● SuperScript III First-Strand Synthesis System, ThermoFisher, 18080051 ● Platinum® Taq DNA Polymerase High Fidelity, Invitrogen, 11304011 ● ExoSAP-ITTM PCR Product Cleanup Reagent, ThermoFisher, 78200.200.UL

# Equipment

Microscopy Pretty much any widefield microscope will work for smFISH. You want to have a good light source, a good camera, and a good objective. For example, we used: Nikon Ti-E automated widefield fluorescence microscope equipped with Plan Apo 1.4 NA 100x objective, automated stage, Pixis 1024BR Cooled CCD camera \(Princeton Instruments), Prior Lumen 220 light source. We use the following filter sets: SP104v2|Chroma|Atto 647N, Cy5|HQ630/20x|Q649LP|HQ667/30m SP105|Chroma|Atto 700|HQ682/12x|Q697LP|HQ721/42m 41028|Chroma|Atto 488/YFP|HQ500/20x|Q515LP|HQ535/30m SP102v1|Chroma|Cy3, TAMRA|HQ546/11x|Q557LP|HQ567/15m Custom filter set|Omega|Cal Fluor 610, Alexa 594|590DF10|610DRLP|630DF30 31000v2|Chroma|DAPI|AT350/50x|400DCLP|D460/50m Note that there are of course many other filters that could work for this; these are just the ones we use. Software For inoFISH probe design, we use the same probe design package developed in the Raj lab for SNP FISH: snpfishdesign. https://bitbucket.org/arjunrajlaboratory/snpfishdesign For inoFISH image analysis, we recommend using the rajlabimagetools software package, which the Raj lab maintains. Depends on MATLAB \(see rajlabimagetools documentation for MATLAB version information). https://bitbucket.org/arjunrajlaboratory/rajlabimagetools/wiki/Home

# Procedure

Core inoFISH protocol This is the basic inoFISH protocol used in several steps of the experimental procedure, below. This protocol is consistent with our in-house inoFISH protocol, available in this Google Doc, which we will continuously update as we tweak experimental conditions as we find them to be useful: https://goo.gl/ujxex1 . The procedure as of May 3, 2017, used for inoFISH experiments in the publication associated with this protocol, is as follows: Pre-inoFISH Cell fixation NOTE: All steps should be done in chemical hood with ventilation. Time required: Fixation: 15 minutes; Permeabilization: at least 1 hour. For multi-chambered slides \(cultured cells): 1. Aspirate off media with vacuum. 2. Add 1 mL PBS per well to rinse, aspirate off. 3. Repeat 1 mL PBS rinse and aspirate. 5 4. Add 1 mL Fixation solution to each well. Allow to fix cells for 10 minutes. Aspirate off fixation solution. a. Keep fixing time consistent between experiments. 5. Meanwhile, label the sample’s plate with the fixing date. Make sure covers and chamber of chambered coverslips are labeled \(to keep track of wells). 6. Add 1 mL PBS per well to rinse, aspirate off. Repeat. 7. Add 1 mL 70% ethanol per well. 8. Parafilm the plate to prevent evaporation of ethanol. a. Store at 4C \(for minimum 1 hour, up to 3 months). inoFISH DAY 1: Hybridization —Prep of probes in hybridization buffer— Time required: 20 minutes, including thawing of probe stocks Per well of sample \(double numbers for 2-well plate): 9. Add 50µL hybridization buffer to Eppendorf tube \(1.5 mL). a. Hybridization buffer is viscous from dextran sulfate, so vortex then spin down 10. Add each of the following to the 50µL hybridization buffer: a. 1 uL editing site detection single oligo probes at a stock concentration of 0.3-1.2 µM \(usually 2 of them at 1µL each, A/I in Cy3/Cy5) b. 1 uL mask oligo \(has common sequence for both SNP probes, added such that mask:probe molar ratio is between 1:1 and 1.5:1; with two detection probes, the range of mask stock concentrations would be 0.6 - 3.6uM) c. 1 uL guide probe \(a typical RNA FISH probe with no tiled oligo at the SNP position, ideally in Cal Fluor 610). For Cal Fluor 610, we use 1uL of 5uM guide probe stock. —Hybridization— Time required: 5-15 minutes, depending on the number of samples 11. Aspirate off 70% ethanol from fixed cell sample well. 12. Add 1

mL wash buffer to each sample well. a. Allow sample to equilibrate for 2-3 minutes. 13. Aspirate off wash buffer and tilt chamber to get residual liquid. a. Be quick so other samples don't dry out. 14. Add 50 µL of probe-containing hybridization buffer as a drop in center of sample. a. Use clean tweezers to place a clean 18 x 18 mm coverslip onto the drop. b. Tap down slide gently with tweezers to spread liquid. 15. Create "moisturizer" using ½ of Kimwipe rolled, tied into a pretzel, and saturated with 750µL of 2X SSC. 16. Replace chamber cover, culture plate cover, and and seal with parafilm. 17. Put in 37°C incubator overnight \(or at least 4 hours). 6 DAY 2: Wash, Anti-fade \(GluOx, Catalase, DAPI), and Imaging Time required: 35 minutes ---Remove Coverslip, 1st wash--- 18. Take out samples from 37°C incubator, remove parafilm. 19. Add 1 mL wash buffer to each sample well. 20. Use clean tweezers to remove coverglass insert. a. Don't crush the sample, discard coverglass insert. 21. Aspirate off wash buffer and replace with 1 mL fresh wash buffer. 22. Replace chamber and plate covers, parafilm. 23. Put back into 37°C incubator for 30 minutes. 24. Start thawing DAPI \(with 10 minutes left to spare). ---2nd wash/DAPI--- Time required: 35 minutes 25. Remove sample from incubator and remove parafilm. 26. Aspirate off wash buffer, add another 1 mL of wash buffer to each sample well. 27. Add 1 uL DAPI \(50 ug/mL stock) to each sample well. 28. Replace covers, parafilm and put back into 37°C incubator for 30 minutes. 29. Meanwhile, set up microscope. ---Glucose Oxidase Prep--- Time required: 10 minutes Glucose Oxidase \(GluOx) is required to reduce photobleaching of Cy5 by dissolved oxygen Glucose + Oxygen in sample --\(GluOx)--> less O2 in sample. Glucose oxidase stock enzyme is aliquoted out and frozen for storage. Note: Glucose oxidase tends to lose potency with repeated freeze/thaw cycles. In order to keep it working as well as possible, either thaw it out right when needed or by putting it at 4C for 10 minutes until ready to add, then immediately refreeze it. After about 20 freeze thaws, it's time for a new aliquot. 30. During 2nd wash, prepare "Anti-Fade" buffer, ~1 mL volume for each Cy5 sample \(for each well → double numbers for 2-well plate). 31. Split anti-fade buffer into 100 & 900 µL volumes for each well. a. 100 µL is for enzymatic solution, 900 µL is just buffer for equilibrating sample. 32. Vortex catalase in its amber vial or aliquots from 4°C. 33. Take out glucose oxidase from -20°C only when ready to add. 34. Add 1 µL of oxidase enzyme and 1 µL of catalase to 100 µL anti-fade buffer. ---Final Wash and addition of glucose oxidase solution \(for Cy5)--- Time required: 5 minutes 35. Aspirate off wash buffer and add 1 mL of 2X SSC at room temperature, wait 1 minute. 36. Aspirate off 2X SSC and add 900 µL of anti-fade \(no enzyme) buffer to each sample well. Allow to equilibrate for 1 minute at room temperature then aspirate. 37. Add 100 µL of Anti-fade with GluOx and Catalase to center of sample. 38. Use clean tweezers to add clean coverslip to lightly squash & reduce O2. 7 ---Imaging--- Time required: usually 10-15 minutes per 100x field of view \(time per sample depends on the number and size of cells you want to capture, as well as exposure times) Sample is now ready for imaging. Imaging can be done over several hours \(store at 4°C in between imaging sessions; at this temperature samples may also be stored overnight with 1mL 2X SSC in the well prior to imaging). If imaging for > 4 hours in a single well, replace that well's 100uL Anti-fade + GluOx and Catalase every 4 hours. As with regular smFISH, to image a cell properly, each image stack should cover the entire body of a cell \(start blurry at the bottom of the cell, and end blurry at the top of the cell). inoFISH image analysis pipeline This is the computational workflow that we use for analyzing inoFISH experiments. It is time-consuming. For an experiment with 200 imaged cells spread over two experimental conditions the full image data analysis pipeline will often take 16 working hours to complete. All image

inspection and spot calling is conducted entirely in the rajlabimagetools software package. We strongly recommend familiarizing oneself with this software and its use in simpler smFISH experiments prior to starting an inoFISH experiment:
https://bitbucket.org/arjunrajlaboratory/rajlabimagetools/wiki/workedExample . The full documentation wiki for rajlabimagetools is here: https://bitbucket.org/arjunrajlaboratory/rajlabimagetools/wiki/browse/ . The SNP FISH analysis pipeline guide is here, and it is directly compatible with inoFISH guide-detection probe colocalization analyses:
https://bitbucket.org/arjunrajlaboratory/rajlabimagetools/wiki/SNPFISH%20Colocalization%20User%20Guide . Please see the rajlabimagetools wiki for a complete description of how to organize your image files and load them into rajlabimagetools. In brief, 1. Cell segmentation 2. Preliminary processing and spot calling 3. Manual inspection of spot intensity thresholds in all channels, with particularly careful attention paid to detection probe channels. a. NOTE: This is the single most time-consuming and difficult step of inoFISH experiments. We have developed multiple tools in rajlabimagetools that allow you to inspect individual fluorescent channels and collections of channels. See the wiki for details. b. We recommend having a second person, ideally someone who also works with smFISH data, review your spot calls. We found this type of analysis review to rapidly improve the quality of detection probe spot calls. i. This will be time-consuming for your colleague, as well. 8 4. Gaussian spot position fitting a. This process refines spot position estimates. 5. Guide-detection spot colocalization 6. Manual inspection of colocalization events with re-review of detection spot calls a. NOTE: This is time-consuming. We again recommend having a colleague review your results as a sanity check. b. Review your spot calls multiple times for consistency. c. Re-threshold spots as needed and update Gaussian fitters/spot colocalizers automatically. 7. Pixel-shift colocalization analysis 8. Extraction and exporting of processed data as required for downstream analyses. Some examples of data tables that you might find useful are demonstrated in worked examples in the wiki:
https://bitbucket.org/arjunrajlaboratory/rajlabimagetools/wiki/SNPFISH%20Colocalization%20User%20Guide . Overall experimental procedure Select a target editing site in a transcript of interest 1. Identify the genomic coordinates of the editing site of interest. 2. Download a RefSeq-annotated reference genomic sequence of the full transcript with the editing site. We will use this for RT-PCR primer design and for inoFISH probe design. We use the UCSC browser tool with the following options: a. Sequence Retrieval Region Options: i. 5’ UTR Exons ii. CDS Exons iii. 3’ UTR Exons iv. One FASTA record per region, 0 upstream, 0 downstream v. Split UTR and CDS exons into separate FASTA records b. Sequence Formatting Options: i. Exons in upper case, everything else in lower case 3. Download reference genome sequence corresponding to the locus around the editing site, about 400-bp upstream and 400-bp downstream. We only need this for gDNA PCR primer design. Use bulk assay techniques, such as gDNA PCR and RT-PCR with Sanger sequencing, to preliminarily check for any editing at this site in biological samples of interest 4. Design gDNA PCR and RT-PCR primers such that your product will contain amplified sequence at the editing site. We recommend designing primers such that your products: a. Have the editing site 100bp - 400bp from both ends of the amplicon \(or from internal Sanger sequencing primer sites). 9 b. Are < 1-kbp in length c. Optional: design additional internal Sanger sequencing primers for each predicted PCR product such that the editing site is still 100bp - 400bp from the primers. 5.

Culture two replicates of a biological sample of interest. 6. Harvest genomic DNA from one replicate your cultured sample of interest. We have used the DNeasy Blood & Tissue Kit \(Qiagen) for cultured human cell lines, but feel free to use whatever works for you. Store at -20C until ready to perform PCR. 7. Harvest total RNA from one replicate of your cultured sample of interest. We have used the miRNeasy Mini Kit \(Qiagen) for cultured human cell lines. Store at -80C until ready to perform RT-PCR. 8. Perform gDNA PCR. We have used Platinum® Taq DNA Polymerase High Fidelity. 9. Perform RT-PCR. a. For RT we have used the SuperScript III First-Strand Synthesis System with included oligo-dT primers. b. For PCR we have used Platinum® Taq DNA Polymerase High Fidelity. 10. Check amplification by running on an agarose gel \(with Ethidium Bromide or SYBR Safe). 11. Eliminate residual dNTPs with ExoSAP-IT. 12. Sanger sequence gDNA PCR and RT-PCR products. 13. Verify RNA editing by comparing Sanger sequencing traces at the editing site in gDNA PCR and RT-PCR products. At the editing site the genomic product should be homozygous A, whereas the RT product should have a mixed A, G trace \(or entirely G if editing is 100% efficient at this site). Check inoFISH compatibility of target editing site 14. Computationally isolate the RT-PCR product Sanger sequencing results for the 81-bp region \(40-bp upstream and 40-bp downstream) of the target editing site. 15. In RT-PCR results, ensure that there are no other nearby heterozygous SNPs, indels, or RNA editing sites leading to transcript base variation near the specific editing site of interest. a. CRITICAL STEP: if the transcript has additional base variation near the particular editing site of interest, it may not be possible to design detection probes \(usually 20-nt - 35-nt) such that the only variation in the detection probe target sequence is the editing site of interest. Design inoFISH guide and detection probes 16. Perform smFISH probe design for the full transcript as previously described *****cit. a. CRITICAL STEP: if the transcript is too short or contains too many repetitive sequences to fit at least 24 oligonucleotide guide probes, then the inoFISH guide may not work, and inoFISH may fail for this target. 10 17. Perform inoFISH detection probe design for the editing site. We use the SNV FISH snpfishdesign pipeline, as previously described. In order to create detection probes that target the unedited and edited versions of the editing site, use a “SNPList” file with a “SNP” at the position \(coordinate on reference chromosome) of the editing site with alleles T/C. a. CRITICAL STEP: it must be possible to design detection probes overlapping the editing site or inoFISH will fail. 18. The inoFISH detection probe design pipeline will provide one detection probe per editing status and shorter probe-complementary masks of three different lengths. inoFISH experiments require at least: both detection probes and one mask. Since the masks are unmodified oligos, we recommend ordering all three options preemptively in the interest of efficient probe set optimization \(discussed below). Screen candidate inoFISH probe sets for optimal signal-to-noise ratio 19. For a small set of combinations of detection probe concentrations and mask length conditions, culture replicates of some simple control samples, for which you have at least RT-PCR with Sanger sequencing as a baseline rough estimate of cell population-wide editing level at the site of interest. a. Note: if you have other estimates of editing level at this site in these samples, such as RT-PCR with restriction endonuclease digestion or high-read-depth RNA-seq data, even better\! 20. Use the above core inoFISH protocol for each candidate probe set concentration and mask length condition. a. Note: If considering more than two probe set conditions, it may make sense to stage FISH experiments over several days, as imaging of detection probe spots often require time-consuming, long exposure times. 21. Complete the above inoFISH image analysis pipeline for each

candidate probe set condition. 22. Compare colocalization rates and pixel-shift false-positive colocalization across probe set conditions. Proceed to the next step of the experimental procedure if you have a probe set condition with an observed colocalization rate and a pixel-shift rate with which you can power an experiment of interest. a. See the associated publication's Supplementary Note for a discussion of the quantitative analysis of inoFISH experiments. b. If none of the probe set conditions produce satisfactory colocalization rates and pixel-shift rates, try a couple other conditions. However, please note that we have encountered a one editing site for which we were unable to generate a probe set after testing six probe set conditions. We are not sure why this site was resistant to probe design 23. Ensure that swapping the dyes conjugated to your optimal probe set's detection probes also gives you a good signal-to-noise \(colocalization-to-pixel-shift) ratio. Conduct inoFISH experiments and editing level analyses 24. Use your optimal probe set in a set of experiments of interest. Make sure that they are powered to detect the effect size for which you are interested in testing. For a discussion of quantitative editing level analysis, see the Supplementary Note of the manuscript with which this protocol is associated.

# Troubleshooting

1. Low colocalization efficiency \(<15%) If not intrinsic to the probe set, which it may be, this may be due to: ● Low detection probe concentration ● Excessively short detection probe channel exposure times on microscope ● Overly conservative detection spot call thresholds ● Increase detection probe stock concentration \(and mask accordingly) ● Increase detection probe exposure times ● Additional re-review of spot calls 2. High pixel-shift colocalization rate \(>25% of colocalization efficiency per detection probe) If not intrinsic to the probe set, which it may be, this may be due to: ● Inappropriately short wash steps ● Excessively low intensity threshold for detection spots ● Short pixel-shift distance Make sure you have precisely timed the wash steps on day 2 of the protocol ● Re-review your detection spot calls; ask a colleague to do the same ● If you are using non-default colocalization distances, you may need to adjust the pixel-shift distance accordingly \(make it at least twice as large as the initialDistance parameter of the colocalizer)

# Anticipated Results

At a minimum, an inoFISH experiment will result in: ● smFISH data on the transcript of interest. For each transcript, inoFISH will give: ● Cell to which it belongs ● Spot position within the 3D image of the cell ■ Nuclear localization if properly counterstained with DAPI ● Editing status label, with frequency equal to the detection efficiency Therefore, an inoFISH experiment will return data on single-cell transcript expression levels in the cells processed, on the editing status of a representative sample of these transcripts, and on the subcellular localization of transcripts with associated editing statuses. Please see the analyses in the manuscript with which this protocol is associated for example results tables extracted from processed inoFISH imaging data. The full repository of those data, with documentation in README files, is here: https://www.dropbox.com/sh/j5umuneita1nck9/AAA4W4I648glUUhePJfXyaRaa?dl=0 .

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