Quantitative mRNA Imaging in Whole Mount Drosophila Brains

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Method Article

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Abstract

This fluorescence in situ hybridization protocol permits detection of the localization and abundance of single mRNAs \(smFISH) in cleared whole-mount adult Drosophila brains. The approach is rapid, multiplexable and does not require molecular amplification; it allows facile mRNA expression quantification with subcellular resolution on a standard confocal microscope. This protocol accompanies Long et al, Nature Methods, published online June 5, 2017 \(nmeth.4309).

Introduction

This protocol describes a simplified, robust whole-mount RNA FISH method for adult Drosophila brains. Optimized processing and clearing help achieve whole brain multiplexed mRNA quantification using a standard confocal microscope. The workflow is as follows: 1) dissection and fixation 2) acetic acid treatment to facilitate probe penetration. Acetic acid results in more consistent results compared to traditional enzymatic digestion based protocols. 3) Autofluorescence quenching using a reductive treatment. 4) Hybridization of the probes to their targets using a high temperature \(50°C) step to enhance diffusion, before lowering the temperature to 37°C for efficient hybridization. 5) Xylene clearing of the tissue. 6) Mounting in an index matched medium, DPX in order to decrease aberrations during imaging. The method does not require molecular amplification, can be performed in two days, and is compatible with immunostaining.

Reagents

Schneider's Insect Medium \(Fisher#S0146) 1x Phosphate Buffered Saline \(PBS) 2% Paraformaldehyde \(PFA) in PBS 0.5% PBT \(1xPBS, 0.5% Triton X-100) 5% \(v/v) acetic acid in water 1xPBS with 1% sodium borohydride Pre-hybr solution \(15% formamide, 2x SSC, 0.1% triton) Hybr solution \(10% Formamide, 2x SSC, 5x Denhard's soln,1mg/ml Yeast tRNA,100µg/ml, Salmon sperm DNA, 0.1% SDS) 30% formamide washing solution \(30% formamide, 2x SSC, 0.06% triton) 2XSSC wash solution \(2x SSC, 0.06% triton) 70%, 50%, 30% EtOH in water Poly-L-lysine coated coverslips \(https://www.janelia.org/sites/default/files/FL%20Recipe%20-%20Poly-L-Lysine%20.pdf) Xylene DPX mountant \(Electron microscopy sciences 13510) FISH Probes: FISH probe libraries can be designed based on transcript sequences using the online Stellaris Designer \(Biosearch Technologies https://www.biosearchtech.com/support/tools/design-software/stellaris-probe-designer). Libraries for each gene typically consist of ~50 probes, but we were able to detect mRNAs with as little as 20 probes in some cases; increasing the number of probes increases the signal to noise ratio. Each probe is 18-22nt long with a 3' end amine-modified nucleotide that we directly couple to an NHS-ester dye \(alexa488, Cy3, alexa594 or Cy5) according to the manufacturer's instructions \(Life Technologies). Free dyes are separated from dye-coupled oligos using the Nucleotide Removal Columns \(Qiagen), and the labeling ratio calculated from the relative absorbance of the dye and DNA using a Nanodrop fluorospectrometer \(ThermoScientific). This approach yields 85-100% of dye-labeling efficiency.

Equipment

Confocal Microscope

Procedure

Day1 Dissection: • 3-5 days old flies are dissected in ice-cold Schneider's Insect Medium. • Dissected tissues are fixed in 2% PFA at 25° C for 55 min. • Fixed tissues are washed with 0.5% PBT, 3x, 10 min each Dehydration: • Dehydrate the fixed tissues with graded EtOH series: 30%, 50%, 70%, 100%, 100%, 100%, 10 min each. • Dehydrated tissues are stored at 4°C with 100% EtOH overnight on a rocker. Day2 Permeation: • Dehydrated tissues are rehydrated with graded EtOH series: 70%, 50%, 30%, 10 min each. • Rehydrated tissues are incubated at 4°C 5% acetic acid for 5 min \(do this in the cold room or on ice). • Wash tissues with 4°C 1xPBS, 3x, 5 min each. • Fix tissues with 2% PFA at 25°C for 55 min. • Wash tissue with 0.5% PBT, 3x, 10 min each. Autofluorescence quenching: • Prepare 1xPBS with 1% sodium borohydride at 4°C \(prepare fresh, use 4°C 1xPBS to prepare the solution). • Incubate tissues in 1xPBS with 1% sodium borohydride for 30 min at 4°C, change solution every 10 min \(do this in the cold room or on ice). • Wash with 4°C 1xPBS for 3x, 5 min each. Pre-Hybridization: • Prepare fresh pre-hybr soln with formamide • Incubate tissues with pre-hybr soln at 50°C for 2 hrs. Hybridization: • Incubate FISH probes in 90°C for 3 min, then snap cold the FISH probes on ice for 10 min. • Prepare ~48µL hybr soln for 1 reaction, which can stain 3-5 brains. • Add 1-2 µL of 50-100ng/µL of FISH probes to the hybr soln \(total reaction value is 50µL) • Incubate at 50° C for 10 hrs, then at 37° C for 10 hrs. Day3 Washing: • Warm up the pre-hybr soln to 37°C. • Add 200µL 37°C pre-hybr soln directly to the hybridization reaction, incubate for 10 min. • Wash with pre-hybr soln, 1x, 37°C, 10 min. • Wash with pre-hybr soln, 1x, 25°C, 10 min. • Wash with 30% formamide washing soln, 2x, 25°C, 30 min each . • Wash with 2xSSC washing soln, 3x, at 25°C, 10 min each • Wash with 1xPBS, 1x, at 25°C, 10 min • Fix tissues at 2% PFA at 25°C for 55 min. • Wash with 0.5% PBT, 3x, at 25°C, 10 min each. Clearing and mounting: • Mount tissues on glass coated with poly-L-lysine. • Dehydrate the tissues with graded EtOH series: 30%, 50%, 75%, 100%, 100%, 100%, 10 min each. • Clear tissues in xylene, 3x, 5 min each \(perform this step in a fume hood) • For confocal imaging, mount tissues in DPX \(Electron microscopy sciences). • For BB-SIM, hold tissues in xylene until imaging.