PIXELBIOSCIENCES GMBH
NovaFISH TISSUE USER GUIDE v0.4

NovaFISH is industry's first multiplexing smFISH (single molecule FISH) technology developed by Pixelbiosciences GmbH. Like conventional smFISH, NovaFISH detects DNA/RNA expression by staining individual molecule and later analyzing with our FREE intelligent cloud-based image analysis platform NovaREAD. The critical made we made with NovaFISH is the autonomous combinatorial color barcoding (Figure 1).

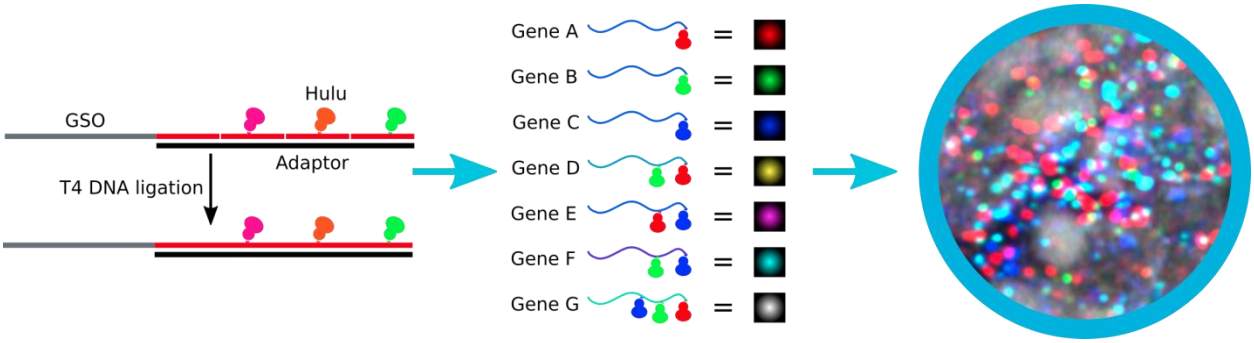

Figure 1. NovaFISH technology. NovaFISH probe is generated via our patented technology, the first enzymatic multiple fluorophore labeling for oligonucleotide pool (left). Currently, up to 7 genes can be barcoded with NovaFISH probe (middle). Each gene will have a distinct combination of 3 base color Nova (Red, Green, and Blue). The composite color dots will become the basis for our barcoding and decoding in multiplexing staining (right).

Each NovaFISH probe in the probe library for a certain gene will be labeled with the same stoichiometry of various fluorophores. The barcoding capacity of NovaFISH is exponentially growing with the number of lasers in the microscope for the imaging. With state-of-art microscope with 7 lasers, one can potentially detect 127 genes in one round of hybridization.

NovaFISH probe can be used to detect DNA/RNA expression in digital quantification. The sample can be in different format, including isolated DNA/RNA, fixed cell, fixed tissue sections, fixed whole mount embryo. This manual is for the detection of RNA/DNA in fixed tissue by NovaFISH.

The following procedures will be divided into 2 major parts: tissue section preparation, and NovaFISH staining.

Tissue section preparation

Step 1 Prepare tissue block

For the cryosectioned sample, snap-freezing your tissue sample as quickly as possible in O.C.T on dry ice (-78 degree, in 3 minutes), or directly in liquid nitrogen equilibrated 2-methyl butane (around -150 degree, 1 minute). Store the

sample in -80 degree before cutting in the cryostat. For FFPE samples, standard fixation with formalin and embedding with paraffin will be acceptable for NovaFISH. Store the tissue block in paraffin at -20 or -80 degree.

Tips

NovaFISH can work with cryo-sectioned frozen tissues or FFPE (formalin-fixed paraffin-embedded) sections. For best RNA quality, tissue blocks should always be stored at low temperature (4 degree or lower) to prevent chemical degradation and RNase mediated degradation of RNA.

Step 2 Sectioning tissue block and mounting

Cut your tissue block with 3-5 um thickness for best NovaFISH staining. Mount them onto 13 mm coverslips with poly-lysine coating or other amine group coating. For FFPE sections, the sample is required to be further baked on 37-degree hot plate for an additional 2 hrs for strong binding to the coverslips. For cryosections, cutting should be done at -20 degree in a cryostat (i.e. cryostat from Leica).

Tips

NovaFISH can work with thicker tissue, like 10-20 um, or even whole mount embryo of platynereis (100 um thick). However thinner tissue sections usually give less background and fewer diffraction artifacts during image acquisition on a microscope.

For cryosectioned tissues, the coverslips should be kept at room temperature for efficient binding with the thin tissue cryosections. When tissues are mounted onto coverslips, these coverslips either should be directly transferred into a 24-well cell culture plate on dry ice or left in the cryostat (around -20 degree) until all tissue sections finished and then transferred into the 24-well plate on dry ice. All sections should be stored at -80 degree before use.

For transportation of these unfixed cryosections to another place, the whole 24-well plate should be sealed properly (with tape) and kept in a box with sufficient dry ice for the whole transportation process. For FFPE sections, they can be transported in 24-well plate at room temperature.

NovaFISH staining can work with tissue mounted on glass slide as well. The staining solution volume might be needed to be adapted to the size of the

coverslip which could cover the sample completely. Sealing the edge of coverslip with rubber cement (i.e. fixogum) will prevent the hybridization solution evaporation, especially at elevated hybridization temperature (37 degree).

Step 3a Fixation (optional for cryosections)

Unfixed cryosections should be fixed in 4% formaldehyde in 1xPBS for 10 min. Then quenching the fixation reaction in 135mM Glycine in 1xPBS for another 10 min. Then another 1xPBS washing for 10 min is required to remove residual formaldehyde and glycine. Finally, the sample should be permeabilized in -70% ethanol overnight at 4 degree.

Tips

All buffers prepared here and after should be DEPC treated (for a detailed protocol of how to make DEPC treated solution, check https://en.wikipedia.org/wiki/Diethyl_pyrocarbonate).

After this fixation step, the RNA in cryosection will be more stable and becoming a bit more resistant to RNase, but still vulnerable to heat-induced chemical degradation of RNA. Therefore the fixed cryosection should still be stored in 70% ethanol at -20 degree. The RNA will be stable from several months to a year. Cryosection sample may be appropriate for transportation at this step. Transportation should be -20 degree with enough 70% ethanol or even higher concentration ethanol. Again the 24-well plate should be well sealed with tape.

Step 3b De-paraffinization (optional for FFPE sections)

Wash the paraffin sections on coverslips with Xylene for 2 x 1 hrs. Then wash out the residual Xylene with 100%, 95%, 70% ethanol gradient, each step 10 min.

Tips

Longer deparaffinization (2 hrs in total) in xylene removes paraffin maximally hence reduce the background staining. Even longer xylene treatment will destroy the sample on the coverslips. RNA retrieval for FFPE sections usually improve the signal. I.e. boiling the section at 80 degree in 0.01M Sodium Acetate pH 6.0 for 30 min.

NovaFISH probe staining

Step 1 Probe Preparation

Resuspend NovaFISH probe mini in 40 ul DNase/RNase Free water (i.e. DEPC treated water or commercial water aliquots for molecular biology use, for NovaFISH midi and maxi, use 200 ul and 500 ul water to resuspend).

Tips

Facilitate the dissolution of NovaFISH probe in water by tapping the tube several times. Alternatively, leave the tube on the bench at room temperature for 20 min. The NovaFISH probe should be stored at -20 degree or lower. It is ok for repeated use by freezing-and-thawing the probe at room temperature. All water used in following steps to prepare the buffer should also be DEPC treated to minimize the RNase contamination.

Step 2 Washing the sections with NovaWash

Before hybridization, wash the coverslip with NovaWash (2xSSC, 2M Urea) 2 times, each time 10 min at room temperature.

Tips

Washing steps could be done on a shaker to have better removal of residual ethanol.

Step 3 Staining with NovaFISH probe

Dilute 0.5 ul of NovaFISH probe in step 1 into 50 ul 1x NovaHyb solution (2xSSC, 2M Urea, 10% dextran sulfate, 5x Denhardt’s solution). Take the NovaFISH working solution on a clean piece of parafilm, then cover the solution with washed coverslip from step 2. Remember the side with tissues should face down to the solution. Hybridize in a humidified chamber at 30 degree for overnight.

Tips

Staining time is minimally 4 hours with NovaFISH. Usually, overnight staining guarantees sufficient staining but also a convenient step for a break in your experiment. The humidified chamber can be easily set up like lock-lock food box in a water bath.

Step 4 Washing the unbound probe

Wash the coverslip in the culture well with NovaWash for 4 times, each time 10 min at room temperature.

Tips

Washing can also be 2 times, 30 min each at room temperature. Or some PIXELBIOSCIENCES users have tried 4 degree overnight washing. And the signal is still well maintained.

Step 5 Mounting

Remove the residual buffer on the coverslip by dipping onto a clean tissue paper. Pipette 10 ul Prolong Gold/Glass mounting solution on a clean glass slide, then immediately cover the mounting solution with the 13mm coverslip having stained cells. Tissue side should be again facing down. Allow the sample to cure for 24-48 hours at room temperature according to the instruction from Prolong Gold/Glass.

Tips

The sample could also be mounted in 2xSSC for immediate imaging.

Imaging the target by epifluorescence or confocal microscope with an appropriate laser. NovaFISH probe is labeled with the combination of Atto488, Atto565, and Atto647N. All fluorophores are barcoded as G (Atto488), Y (Atto565), and R (Atto647N). Check your probe barcoding scheme on the tube label. For example, Gapdh-1G1Y1R is standing for mouse Gapdh NovaFISH probe with one Atto488, one Atto565, and one Atto647N. GAPDH-2G1R is standing for Human GAPDH gene with 2 Atto488 and one Atto647N.

Appendix 1 Recommended Reagents from other vendors

Name	Vendor	Cat. No.
Prolong Gold	ThermoFisher Scientific	P10144
Prolong Glass	ThermoFisher Scientific	P36982