BIOSEARCH TECHNOLOGIES
Advancing Nucleic Acid Technology℠

Improving Dye Brightness and Photostability in Stellaris® RNA FISH Assays

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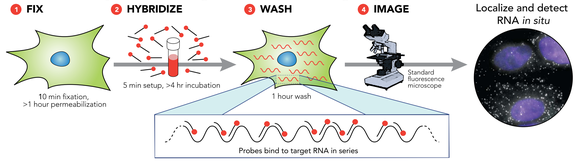
STELLARIS RNA FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH) ASSAY

- Allows visualization and quantification of single molecules of RNA in fixed adherent cells and in tissue.
- Multiple oligonucleotide sequences are each appended with a fluorescent label.
- Probes collectively bind to a target transcript creating bright spots.



Cells are fixed on coverslips and probes sets are hybridized for 4 to 16 hours at 37 °C in 50 µL of hybridization solution (10% dextran sulfate, 10% formamide in 2X SSC). Samples are then washed and the nuclei are stained with DAPI. A mounting media is then added and the coverslip is sealed with clear nail polish.

DYE BLINKING AND BLEACHING

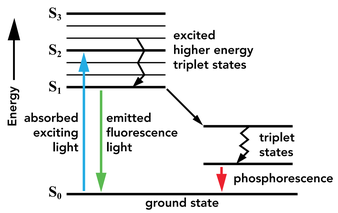
Photobleaching is permanent loss of color or fluroescence upon exposure to light and occurs mainly by
i) Photo-oxidation – reactive singlet oxygen is formed via sensitization by triplet state dyes.
ii) Photoionization – formation of dye radicals from higher excited states.

Photobleaching occurs with all dye classes:

- Fluorescein (FAM, FITC)
- Cyanines (Cy™5, Quasar® 670 dye, Cy3, Quasar 570 dyes)
- Rhodamines (TAMRA, CAL Fluor® dye)

An antifade can prevent irreversible photobleaching of dyes by:

- Removing oxgen that is dissolved in the buffer.
- Quenching the dye triplet state and restoring the dye to the ground state.
- Acting as an antioxidant and promoting recovery of photoionized dye radicals.



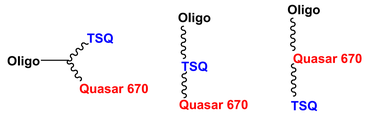
Jablonski Diagram: shows electronic states of a molecule and transitions between them.

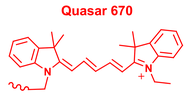
- Triplet states are long-lived.
- When a dye is trapped in a triplet state, it blinks.

COVALENT TSQ (TRIPLET STATE QUENCHERS)

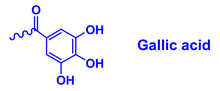
Conjugation of triplet state quenchers adjacent to cyanine fluorophores (such as Cy5 and Quasar 670 dyes) can be more effective at preserving and enhancing fluorescence intensity than adding TSQs diffusionally.

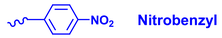
Several TSQ – Quasar 670 oligo configurations have been tried:





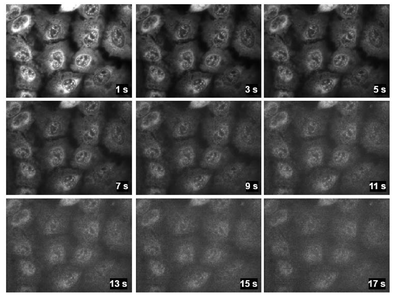
TSQ molecules



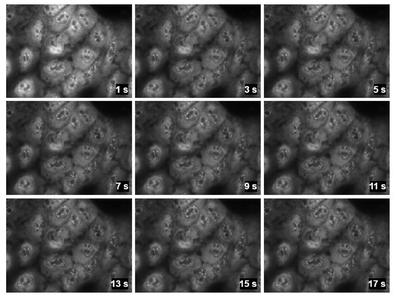


Nitrobenzyl was conjugated adjacent to a Quasar 670 moiety and appended to a T_{30}. A reference Quasar 670-T_{30} sample was also prepared. These probes bind to cellular poly-A RNA tails. The images below were recorded at two second intervals.

3' Quasar 670 - T30 MINUS GLOX buffer



Quasar 670 TSQ - T30 MINUS GLOX buffer



Next, a GAPDH Stellaris probe set was prepared with the nitrobenzyl-Quasar 670 adduct. Unfortunately, compared to a standard GAPDH Quasar 670 probe set, consistent spots of mRNA were not seen throughout the slide. This suggests that the TSQ moiety is too hydrophobic and interfered with target hybridization.

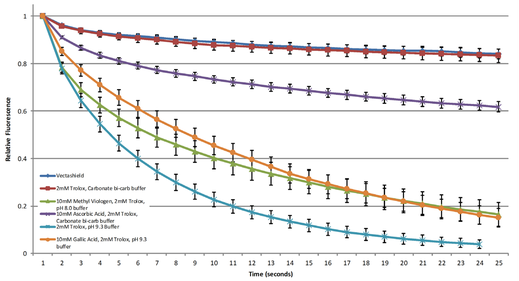
DIFFUSIONAL ANTIFADE SOLUTIONS

GLOX (glucose oxidase) is a widely used antifade that is added to buffer solutions.
Glucose oxidase catalyzes: β-D-glucose + O_2 → D-gluconolactone + H_2O_2
A Hydrolysis reaction lowers the pH of the solution: D-gluconolactone + H_2O → gluconic acid
Catalase is added to remove reactive hydrogen peroxide: 2 H_2O_2 → 2 H_2O + O_2
*** GLOX is incompatible with pH dependent dyes such as fluorescein.

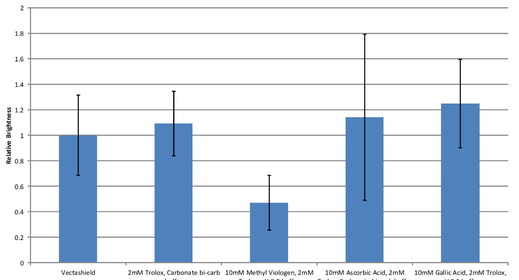
Adding GLOX to a solution with FAM makes the fluorescence disappear.

- Dye bleaching occurs within seconds with FAM and Quasar 670 dyes under standard wide field microscopy conditions using a metal halide lamp.
- Several potential triplet state quenchers and antioxidants were added to the final mounting buffer solution to test antifade efficacy.
- Dye fluorescence intensity over time (photostability) and initial fluorescence intensity (brightness) were calculated for both FAM and Quasar 670 dyes.
- The antifade efficacy depends on several parameters including concentration, pH, viscosity, and buffer composition.
- Vectashield® from Vector Laboratories is a very effective antifade for both FAM and Quasar 670 dyes.
- Some other antifade compositions were almost as effective as Vectashield, but not as reproducible.

GAPDH FAM Photostability



GAPDH FAM Brightness



CONCLUSIONS

- Both diffusional antifades and covalent TSQ can improve dye brightness and photostability.
- The covalent TSQ moieties worked well in test poly-T sequences but appeared to interfere with hybridization in a Stellaris assay with a mixed base probe set.
- Efforts are ongoing to find an antifade as effective and reproducible as Vectashield.

REFERENCES

- M. Batish, A. Raj, S. Tyagi, Single molecule imaging of RNA in situ, *Methods Mol. Biol.*, **2011**, *714*, 3-13.
- R. B. Altman et al., Cyanine fluorophore derivatives with enhanced photostability, *Nature Methods*, **2012**, *9*, 68-71.
- M. Levitus, S. Ranjit, Cyanine dyes in biophysical research: the photophysics of polymethine fluorescent dyes in biomolecular enviornments, *Quarterly Reviews of Biophysics*, **2011**, *44*, 123-151.

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