Blue Nuclear Label Red-shift

Sometimes the bleed-through of the blue nuclear label into the green channel seems higher than usual. There is a real phenomenon behind this increase; it's called photoconversion.

It is known for the blue nuclear labels DAPI, Hoechst and Vybrant DyeCyle Violet, but can happen to other labels too. Red-shifts of up to 80 nm have been observed in real samples, e.g. cy5-Beta-catenin on polyvinylidene (PVDF) membrane.

Note that the in-solution values stated in reference may differ from spectra in other conditions.

DAPI, Hoechst 33258 and Vybrant DyeCyle Violet Exhibit Photoconversion

- Photoconversion by exposure to UV light
    - The 405 nm laser is far enough from the UV to not cause this
- Photoconverted derivatives have low photostability
    - Bleached easily
    - Photobleaching of DAPI & Hoechst 33258 recovers 50% in 60 min
    - Photoconverted derivatives bleach at the same rate
- The photoconversion is reversible
    - After the photoconversion reversal an equilibrium state lasts for at least 2 more hours
- The photoconversion is dose dependent
    - The dose response of DAPI is stronger
    - 4x increase in 100 s
    - (Hoechst 2x in the same time)
- Hydroxen peroxide induces similar photo-oxidation
    - Red-shift
    - Takes 4 h for Hoechst
    - 1.5 h for DAPI
    - Reversible

Spectral Characteristics of the Red-shifted Label

- Excitation at 450-490 nm
- Emission at 480-600 nm
    - Maxima at 540 nm

Too High Label Concentrations Can Diminish Signal

- High concentrations of DAPI & Hoechst 33258 diminish blue signal
- Self-quenching
- Signal in RNA increased

DAPI Spectral Shift

- DAPI binding with DNA is solution specific
- It can change from groove-binding to intercalation
- It can also induce polymer-dye adducts
- Emission maximum blue-shifts when complexed with DNA

DAPI Spectral Characteristics

- Excitation maxima at 364 nm
- Emission maximum at 454 nm

DAPI Red-shift Conditions

- DAPI also binds to RNA
- Emission maximum red-shifts when bound to RNA
    - Binding to RNA minor groove
    - Complexation with polyA-polyU
- Dissociation 100 times faster than from DNA
- High dye/phosphate ratio (1:10) reveals a new binding form
- New emission maximum 540 nm
- Binding of DAPI to sites in polynucleotides in proximity to previously bound DAPI
    - Disappears if background electrolyte is 0.4 M KCl
    - Distrupts DAPI-DAPI electrostatic interactions
    - DAPI-polyadenylic acid (polyA)
    - DAPI-Polyphosphate (polyP)
        - Excitation 415 nm
        - Emission 550 nm
    - Inositol phosphates (IP5 & IP6)
        - Emission 550 nm
    - Heparin

        - Emission 550 nm
    - Amorphous calcium phosphate (ACP)
        - Emission 550 nm
        - Not Hydroxyapatite (HAp)
- “Red-shifted DAPI fluorescence is not due to specific substrate chemistry, but indicates the presence of a high density of negatively-charged surfaces or molecules that locally concentrate DAPI. This local, increased DAPI concentration enables DAPI–DAPI interactions and its resultant red-shifted fluorescence.”
- High concentrations of glycerol (as mounting media) are proportional to the level of DAPI photoconversion
- DAPI photoconversion rate has two components
    - Fast half-life < 10 s
    - Slow half-life > 60 s
    - Both are UV illumination intensity dependent (non-linear)

Hoechst 33258 Red-shift

- Exposure to UV causes both photobleaching and photoconversion
    - New emission maxima 540 nm
- Protonation of the dye
    - Also by exposure to hydrogen peroxide
- Similar properties in acidic environments (pH 0.5-3.0)
    - Quantum yield drops 80-fold
- QY increased 20-fold in pH 4.5
- Acid treatment reversible
- Equilibrium at 60 min past UV exposure
- Red-shifted protonated form shows up in nucleoli
    - RNA-binding
        - Excitation 369 nm
        - Emission 437 nm

Hoechst Spectral Characteristics

- Excitation 355 nm
- Emission 465 nm

Vybrant DyeCyle Violet

Vybrant DyeCyle Violet also exhibits the photoconversion red-shift of it's spectra.

Mitigating the Red-shift

- Minimize UV intensity when imaging the blue nuclear labels.
    - Total dose or exposure time
- Use low label concentration
- Use low glycerol concentration in mounting media.
- Acquire blue nuclear channels last to minimize green channel false positives
- Use alternate nuclear dyes like DRAQ5 or RedDot which fluoresce in the far red region

Sources

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