Epicocconone™, a cell-permeable fluorophore for staining cytoplasmic compartments of live cells

Key words: epicocconone, fluorescence, microscopy, multiplexing

Introduction

Fluorescent labeling of sub-cellular structures can be achieved using a variety of fluorescent stains[1, 2], fluorescently-labeled antibodies[3] or co-expression of a GFP-fusion protein[4, 5]. Generally cells must be permeabilized to allow entry of the fluorescent stain or labeled antibody or genetically altered to in order to express GFP. It is often impossible to wash away excess label from intracellular compartments or from inside tissues, resulting in high background. Some stains are also cytotoxic or affect cellular processes limiting their uses for live cell imaging. The ideal fluorescent stain for intracellular imaging would be cell permeable, non-toxic, readily distinguished from unbound stain and able to be multiplexed with other common stains (e.g. by having a long Stokes' shift).

Epicocconone is a recently discovered, low molecular weight (MW 410), water-soluble, fluorescent natural product from the fungus *Epicoccum nigrum*[6] that fulfills many of these criteria and which has potential in cellular staining. This compound spontaneously, and reversibly, conjugates to lysine residues in proteins yielding an intensely fluorescent orange/red product that is easily distinguished from unconjugated stain. In hydrophobic environments, such as within cell membranes and hydrophobic pockets in proteins, the quantum yield of this fluorophore also increases markedly.

Epicocconone is excitable by common lasers such as violet GaN (400-410 nm), argon Ar ion (488 nm), frequency doubled Nd:YAG (532 nm) and He-Neon (543 nm), enabling analysis by standard fluorescence instrumentation (fluorescence and confocal microscopy, flow cytometry, etc).

This application note describes the use of Epicocconone™ for staining cytoplasmic structures of live mammalian cells for confocal fluorescence microscopy. The stain is non-toxic, stains cells fluorescent orange, and can be multiplexed with other fluorophores such as blue-emitting Hoechst 33342 or green-emitting SYTOX.

Materials and Methods

Reagents

1. Epicocconone™ in solution (2.4 mM, 1 mg epicocconone /mL dimethyl sulfoxide, FLUOROtechnics, http://www.fluorotechnics.com/epic.asp)
2. SYTOX-green dye solution (5 mM in DMSO, Molecular Probe, Oregon, USA, Catalog No.: S-7020)
3. Hoechst 33342 solution (8 mM in H_2O, Molecular Probe, Oregon, USA, Catalog No.: H-1399)
4. Growth medium and staining solution: Dulbecco's Modified Eagle Medium (DMEM,GIBCO, NY, USA, Catalog No.: 10569-010) containing 10 % FBS
5. Mounting medium: 50 % glycerol in 20 mM phosphate buffer (pH 8.5)
6. Phosphate buffered saline (pH 7.4 ± 0.2; Sigma-Aldrich, Sydney, Australia, Catalog No.: P-4417)

Equipment

1. Tissue culture flask (75 cm^2, COSTAR, MA, USA, Catalog No.: 3275)
2. Tissue culture multi-well plate (3.5 x 1.0 cm, Flow Laboratories, Virginia, USA, Catalog No.: 76-058-16)
3. Tissue culture multi-well plate (6.0 x 1. 5 cm, Flow Laboratories, Virginia, USA, Catalog No.:76-037-05)
4. CultureWell™ Chambered Coverglass for cell culture (Molecular Probe, Oregon, USA, Catalog No.:C37000)
5. Live cell chamber & 0.16 – 42 mm cover glass (POC chamber System, H.Saur Laboratory need, Reutlingen, Germany, Catalog No.:15-6108-381)
6. Tempcontrol 37-2 digital (Leica Microsystems, Heidelberg GmbH, Germany, Catalog No.:0503.000)
7. TCS SP2 Confocal Laser Scanning Microscopy with DM IRE microscope (Leica Microsystems, Heidelberg GmbH, Germany)
8. FACSCalibur (BD Biosciences, Sydney, Australia)

HCT 116

HCT 116 (APAF, Australia) cells, a human colon cancer cell line were maintained in tissue culture flasks containing DMEM (20 mL) at 37°C in 5 % CO_2. Actively growing cells that are ~ 80 % confluent were used for cytotoxicity test and epicocconone-staining.

Cytotoxicity test

HCT 116 cells were cultured as described above and were seeded in duplicate at cell densities of approx. 2 x 10^5 cells per well in tissue culture multi-well plates (3.5 x 1.0 cm) containing 2.5 mL of DMEM growth medium containing various final concentrations of Epicocconone™ up to 12 µM. The cells were grown for periods up to 24 hours, and the cell numbers were determined by counting the cells that were not taking up trypan blue stain using Hemocytometer at 0.5, 0.5, 1, 2, and 24 hr intervals. Identical cultures without stained with Epicocconone™ were used as controls.

Staining methods

Live cells

Staining live cells with Epicocconone™ was performed in a temperature-controlled POC chamber system (H. Saur, Reutlingen, Germany). HCT 116 cells were grown as a monolayer on cover glasses (42 x 0.16 mm, POC Chamber System) placed in a tissue culture multi-well plate (6.0 x 1. 5 cm) containing 2.5 mL DMEM growth medium. One cover glass with the cell monolayer was aseptically transferred and placed into the POC cell chamber, and a dye-free growth medium (~ 2 mL) was added to the chamber. The cells were observed using a DM IRE microscope. The temperature of the growth chamber was maintained at 37 °C (Tempcontrol 37-2 digital) during the assay time. Immediately prior to the staining, the cells were washed twice with PBS and then incubated with 2 mL of growth medium containing Epicocconone™ (final conc. 6 –12 µM) over 30 min to obtain real time live cell images (TCS SP2).

Fixed cells

HCT 116 cells were grown as a monolayer in a CultureWell™ Chambered Coverglass (~10^5 cells/mL/well). Immediately prior to staining, the medium in the chambered coverglass was carefully decanted. The cells were washed twice with PBS by pipetting, fixed in chilled (4 °C) 1 x PBS containing 4 % formaldehyde for 20 min at 4 °C, and washed twice with 1 x PBS. The chambered coverglass was filled with 200 µL of DMEM growth medium containing Epicocconone™ (final conc.: 12 – 24 µM) for 30 min-staining at RT (dark). Dual staining was achieved by adding SYTOX-green (final conc.:1.25 – 2.5 µM), or Hoechst 33342 (final conc.:1 – 4 µM).

The chambered coverglass with stained cell samples was mounted in a glycerol-based mounting medium onto a clean glass slide (Product Information for CultureWell™ coverglass, x 37000).

Confocal Laser Scanning Microscopy

Images of fluorescently stained cells were obtained using a Leica TCS SP2 confocal microscope equipped with a 405 nm diode laser (for Hoechst 33342) and a 488 nm ArKr laser (for Epicocconone™ and SYTOX). Samples were viewed under a Leica DM IRE2 microscope (Leica Microsystems, Germany). Stained cells were imaged using a 63 x or a 100 x lens. In order to ensure uniformity for comparisons of fluorescent staining, all cell samples were analyzed the same day with the same laser scanning settings.

Image series of live cells stained with Epicocconone™ were recorded using time image series (xyt) (Leica Microsystems, user manual or http://www.univ-rouen.fr/inwerm-u413/ifrmp/tutorials/zeitbildserien_en.htm). Emission spectra of Epicocconone™, SYTOX and Hoechst 33342) were obtained using recording lambda series (xyλ) (Leica Microsystems, user manual or http://www.univ-rouen.fr/inwerm-u413/ifrmp/tutorials/zeitbildserien_en.htm). Data from all

images were processed using the Leica Confocal Software (Leica Microsystems, Heidelberg GmbH).

Results

Effect of Epicocconone™ on growth of cells

Epicocconone™ was tested for its effect on viability of a mammalian cancer cell line, HCT 116. Cell numbers of Epicocconone™-stained sample were counted by flow cytometry, and compared with those of unstained cells. Epicocconone™ had no significant effect on the growth rate of a human colon cancer cell line HCT 116 over 24 hours at a concentration of 12 µM, which is similar to or higher than typically used for live cell staining (Fig. 1).

Live and fixed cells staining

Epicocconone™ was used for staining both live and fixed HCT 116. Epicocconone™, a small neutral molecule, freely diffused across the plasma membrane, and stained both live and fixed mammalian cells fluorescent orange (fig.2) without the need for permeabilizing agents that are required for antibody linked or small ionic fluorophores.

Epicocconone™ (6 – 12 µM) stained the plasma membrane of live cells fluorescent orange almost immediately (less than 1 min). This stain appears to readily permeate the plasma membrane as cytoplasmic compartments were typically stained within 30 min (http://www.gelcompany.com/epic.asp). An animation file showing Epicocconone™-staining in real-time can be viewed at http://www.gelcompany.com/epic.asp.

For fixed cell staining, Epicocconone™ was used at a concentration of 24 µM. Epicocconone™ stained most of the cytoplasmic compartment of fixed cells bright orange and it did not stain nucleic acid components as indicated by counter staining with Hoechst 33342 and SYTOX (Fig. 3).

Multiplexing

Nucleic acid stains, e.g. blue-emitting Hoechst 33342 and green-emitting SYTOX were used as multiplexing pairs with Epicocconone™.

The xyλ scan generated an emission spectrum of Epicocconone™ ranged from 530 to 680 nm, with a maximum peak at 584 nm. Maximum emission peaks recorded by the xyλ scan for both nucleic acid stains are 479 nm (408 – 670 nm) for Hoechst 33342 and 524 nm (494 – 596 nm) for SYTOX.

The long Stokes' shift of Epicocconone™ (λex = 395 or 495 nm; λem = 584 nm) stained cells makes this stain a useful partner for both blue emitting fluorophores (e.g. Hoechst 33342; λem 479 nm) and green emitting (e.g. SYTOX; λem = 524 nm) fluorophores as there is very little spectral overlap between Epicocconone™ and either of the other two stains.

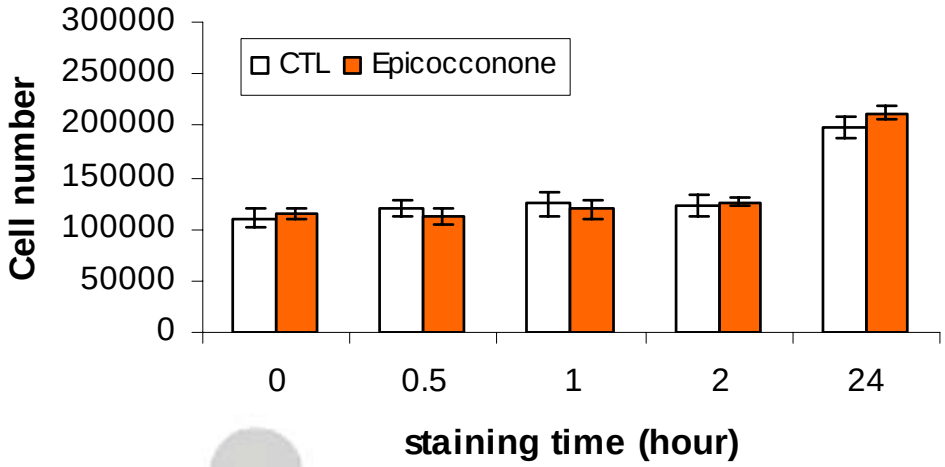

Figure 1. Low cytotoxicity of Epicocconone™ during its staining HCT 116 cells over 24 hours.

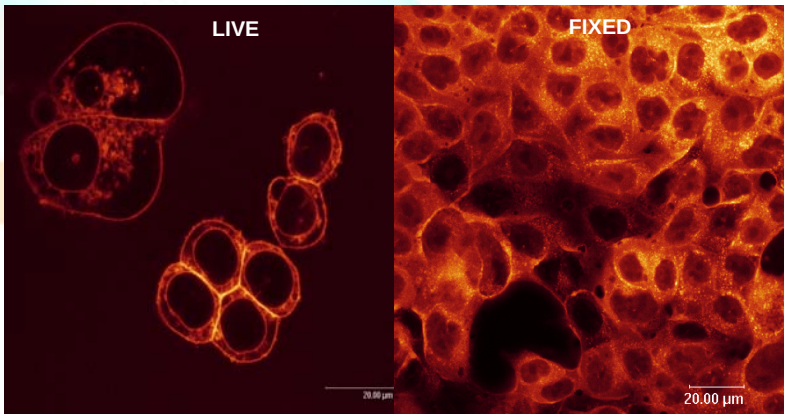

Figure 2. Confocal image showing Epicocconone™ staining both live (37 °C; 30 min) and fixed cells (25 °C30 min) fluorescence orange. Images were obtained using Leica Confocal equipped with (488-nm argon ion laser). Bar: 20 µm.

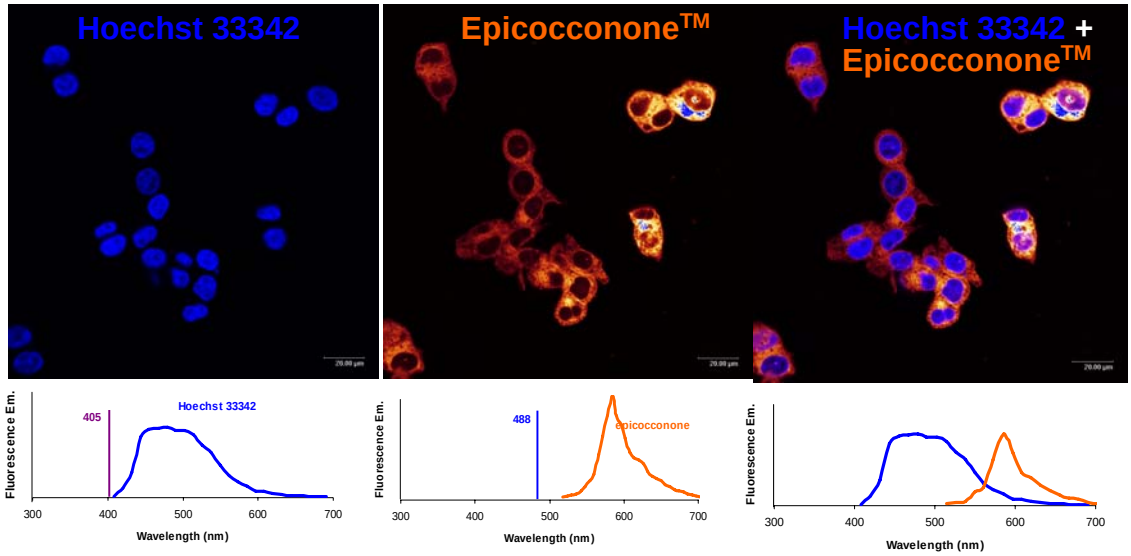

Figure 3-1. Images of HCT cells stained with Hoechst 33342 and Epicocconone™ and emission profiles. Cells were fixed and dual stained with Hoechst 33342 (DNA) and Epicocconone™. Images of Hoechst 33342- and Epicocconone™-stained cells were separately scanned by two wavelength-lasers (405 nm for Hoechst and 488 nm for Epicocconone™). Bar:16 µm.

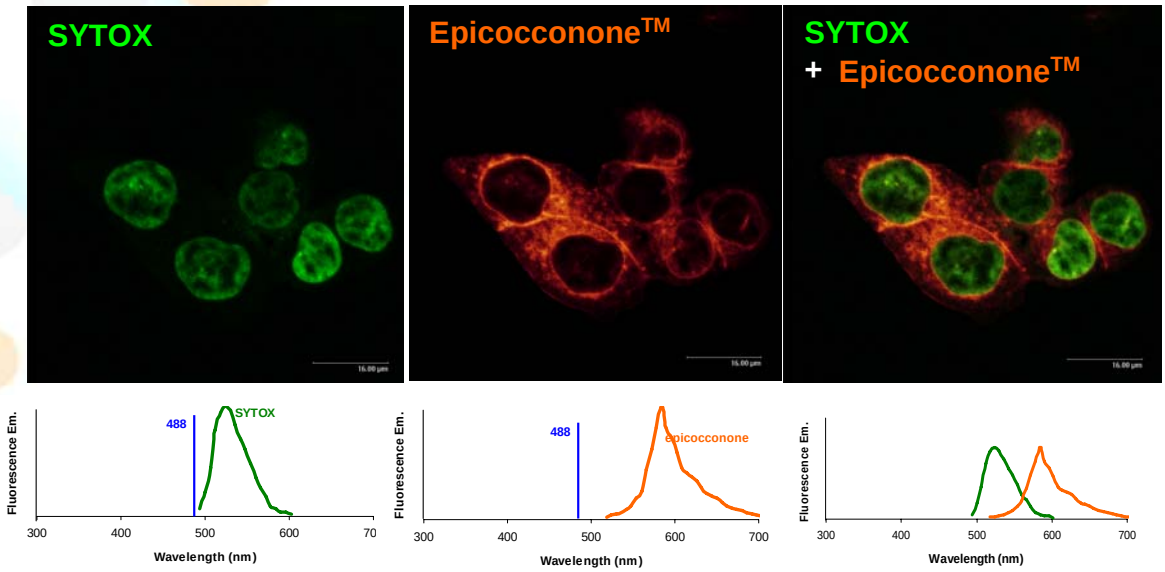

Figure 3-2. Images of HCT cells stained with SYTOX and Epicocconone™ and emission spectrum profiles. Cells were fixed and dual stained with SYTOX-green (nucleic acids) and Epicocconone™. Images were obtained using a single laser (488 nm argon laser) scanning of a cell sample in Leica Confocal TCS SP2 (channel 1 for green-emitting SYTOX; channel 2 for orange-emitting Epicocconone™). Bar: 16 µm.

Conclusions

Epicocconone™ has a number of useful characteristics for cellular imaging;
1) The spectral characteristics (both emission maxima and quantum yield) of the fluorophore change significantly when cells are stained. This enables cells to be brightly stained against a very low fluorescence background without the need to wash away unbound fluorophore.
2) Live cells are readily permeable to Epicocconone™ and do not require any pre-treatment to allow the stain to be taken up.
3) Epicocconone™ does not affect the growth rate of a wide range of cell types (bacteria, yeast, mammal; results for bacteria and yeast not shown) at concentrations similar to those used for staining.
4) Epicocconone™ is excited by a variety of light sources used in standard fluorescence based instrumentation.
5) The long Stokes' shift of Epicocconone™ makes it ideal for use with a wide variety of common short Stokes' shift fluorophores in multiplex assays.

The technical features of Epicocconone™ suggest it will have wide utility as a fluorescent stain for cellular imaging.

Reference

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