Multiplexing with near-infrared (NIR) dyes

Exploiting the NIR spectrum using the CellInsight CX7 High Content Analysis Platform and Alexa Fluor 750 secondary antibodies

Introduction

Researchers are commonly faced with analyzing cells already expressing both green and red fluorescent proteins. Since the blue channel of an imaging system is routinely occupied by a nuclear stain for automated analysis, it can be problematic to image with an additional channel for immunodetection. A red fluorescent protein such as TagRFP occupies both orange and red channels, while the tail of its protein fluorescence may be detected in the deep-red channel. Adding a deep-red fluorophore will cause further bleed-through if the red excitation filter excites the fluorophore.

Moving to NIR

The Thermo Scientific™ CellInsight™ CX7 High Content Analysis Platform offers an NIR channel for either wide-field or confocal imaging (Figure 1). This channel is spectrally well separated from the deep-red channel and enables an optimal combination of green and red fluorescent proteins together with standard immunocytochemistry. Since a red fluorescent protein occupies both orange and deep-red channels (Figure 2), moving into the NIR spectrum using Invitrogen™ Alexa Fluor™ 750 secondary antibody conjugates offers greater resolution in the red spectrum for easier multiplexing (Figure 3). With the appropriate fluorophore selection, the CellInsight CX7 High Content Analysis Platform can be used to image up to 5 colors in a confocal image to extract additional biological information from a sample.

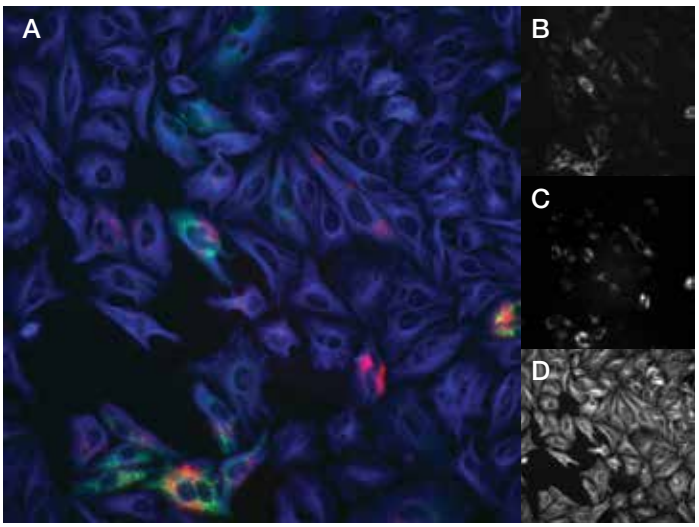

Figure 1. Multiplex imaging on the CellInsight CX7 High Content Analysis Platform. (A) Composite image of U2OS cells expressing **(B)** Invitrogen™ CellLight™ Mitochondria-GFP and **(C)** Invitrogen™ Premo™ Autophagy Sensor RFP-p62 (mKate2), and labeled with **(D)** anti-tubulin antibody and Invitrogen™ Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor™ 750 conjugate.

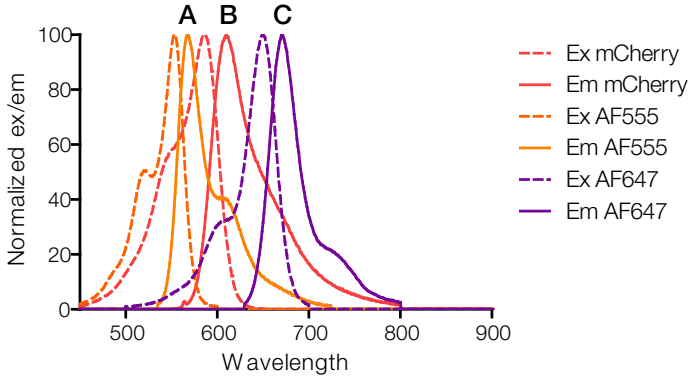

Figure 2. Fluorescence chart of overlapping fluorophores in the red spectrum. A red fluorescent protein (e.g., mCherry) restricts use of the orange and deep-red channels for multiplexing. A = Invitrogen™ Alexa Fluor™ 555 dye, B = mCherry, C = Invitrogen™ Alexa Fluor™ 647 dye.

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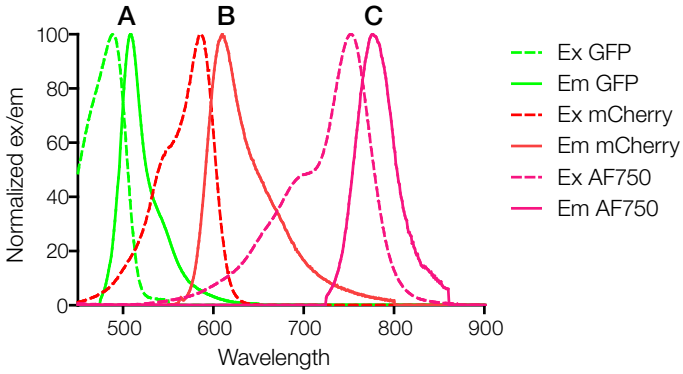

Figure 3. Fluorescence chart of spectral resolution using a NIR dye to multiplex. A red fluorescent protein (e.g., mCherry) is spectrally resolved from GFP and NIR dyes for easy multiplexing and quantitation. A = emerald GFP, B = mCherry, C = Alexa Fluor 750 dye.

Five-color imaging

Confocal imaging and multiplex analysis was performed to explore the impact on cell health when autophagy is blocked using chloroquine (Figures 4 and 5). U2OS cells stably expressing the EGF receptor as a GFP chimera were plated in a 96-well plate. The following day, cells were incubated with 10 µM EdU. Following fixation and permeabilization, cells were labeled with Hoechst 33342 and the Invitrogen™ Click-iT™ Plus EdU Alexa Fluor™ 647 Imaging Kit and incubated with mouse anti-tubulin antibody and rabbit anti-LC3B antibody followed by Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 750 conjugate and Invitrogen™ Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 555 conjugate. Plates were imaged on the CellInsight CX7 High Content Analysis Platform using a 20x air objective lens and the blue, green, red, far red, and NIR filter sets. The results indicate that the full spectral breadth of the CellInsight CX7 High Content Analysis Platform can be used for easy multiplexing and quantitation in a variety of imaging applications.

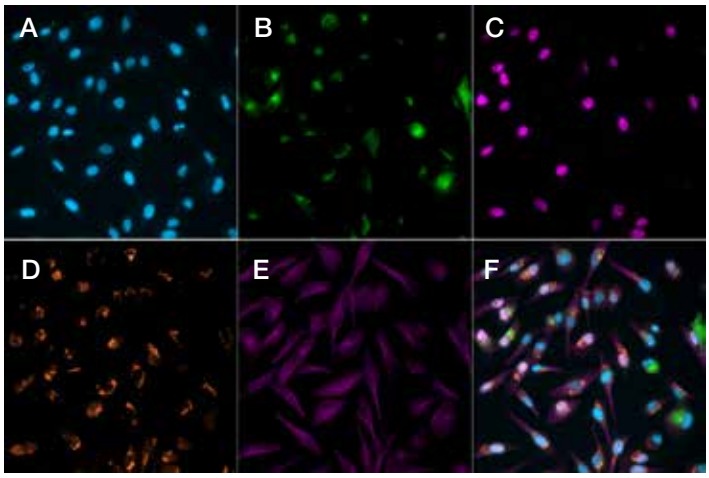

Figure 4. Five-color imaging on the CellInsight CX7 High Content Analysis Platform. (F) Composite image of U2OS cells stably expressing **(B)** the EGF receptor as a GFP chimera. Cells were treated with chloroquine to block autophagy and incubated with EdU prior to fixation. Cells were labeled with **(A)** Hoechst™ 33342 dye, **(C)** anti-LC3B antibody and Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 555 conjugate, **(D)** the Click-iT Plus EdU Alexa Fluor 647 Imaging Kit, and **(E)** anti-tubulin antibody and Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 750 conjugate.

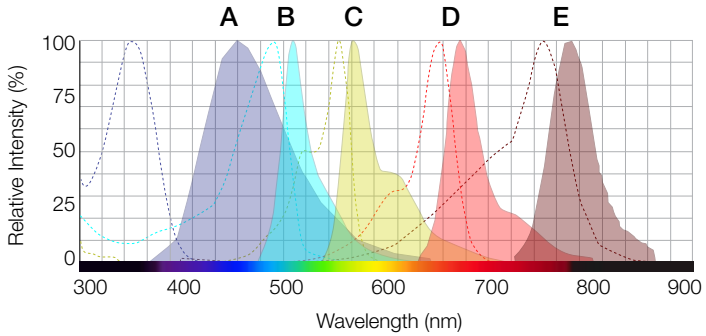

Figure 5. Fluorescence SpectraViewer chart of a 5-plex assay. A = Hoechst 33342, B = emerald GFP, C = Alexa Fluor 555 dye, D = Alexa Fluor 647 dye, E = Alexa Fluor 750 dye.

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