Using near infra-red dyes to deal with autofluorescence background in biological samples

Application Note

When the aim of an experiment is to image single molecules, autofluorescence background can impede their detection. In cells, a major contribution to the fluorescence background is from autofluorescence from within the cell. Commonly, evanescent field excitation or Total Internal Reflection Fluorescence (TIRF) is used to reduce the background, however, this method limits excitation to a thin layer a few hundred nanometers thick and is unable to follow molecules as they are internalised or to image organelles within the cell. A second approach which is spectral involves exciting fluorescence in the near infra-red (NIR) region of the spectrum, where the background generated by cellular autofluorescence is much lower. Dr. Stephen Webb and his research team at the Central laser facility, Didcot, UK have investigated the brightness, photostability and blinking characteristics of a range of commercially available organic NIR dyes under the same conditions with a view to selecting the most appropriate for single molecule imaging in cells.

Dyes compatible with NIR imaging and that could be excited at 695 nm or 780 nm and MCF-7 cells, a commonly used breast cancer cell line that produces a noticeable autofluorescence background when illuminated with visible wavelength light were selected for the investigation.

Obtaining an adequate signal is paramount when selecting dyes for single molecule imaging and hence, Dr. Webb and his team chose to investigate the four brightest NIR dyes based on initial ensemble fluorescence studies. The dyes selected were Alexa 700 and IRDye 700DX (excited at 695 nm) and Alexa 790 and IRDye 800CW (excited at 700 nm). Single molecule intensity, photobleaching rate and blinking characteristics were the parameters investigated for each dye molecule. The results of this study showed that both IRDyes had mean intensities superior to the corresponding Alexa dye, although the difference between Alexa 700 and IRDye 700DX was minimal. Concerning photobleaching, IRDye 700DX was more photostable than Alexa 700, whereas IRDye 800CW and Alexa 790 displayed similar photobleaching kinetics.

To investigate the contribution of blinking to the measured photobleaching kinetics, histograms of fluorescence emission periods were produced from the same datasets. A single exponential decay curve was fitted to these histograms to obtain the mean time that each dye continuously emitted before entering the dark state (the ‘on-time’). The duty cycle for each molecule was defined as the summed emission periods divided by the time elapsed when the final bleaching event occurs. Alexa 700 had an average ‘on-time’ of less than one second and a duty cycle of 0.62, i.e. Alexa 700 intensity traces comprise short bursts of fluorescence with dark periods lasting several seconds. In comparison, IRDye 700DX had an average ‘on-time’ of 1.4 seconds and a higher duty cycle, indicating that the dye emits for longer periods, punctuated by brief periods in a dark state. Of the longer wavelength dyes, it was found that IRDye 800CW had a marginally longer ‘on-time’ and a greater duty cycle than Alexa 790.

The four NIR dyes under investigation were further tested by imaging in MCF-7 cells. Initially unlabelled MCF-7 cells were imaged and a decrease in the autofluorescence background was observed as expected with increasing wavelength. When cells were excited at 780 nm almost no background was observed above the detector noise (Fig 1 A). Labelling cells with transferrin-IRDye 700DX and transferring-IR Dye 800CW revealed that the total background intensity reduces with increasing wavelength with both epi and TIRF illumination (Fig 1 B). Additionally, it was discovered that IRDye 700DX was not bright enough for epi-fluorescence illumination but IRDye 800CW maintained a sufficient signal to background ratio when imaged in this mode.

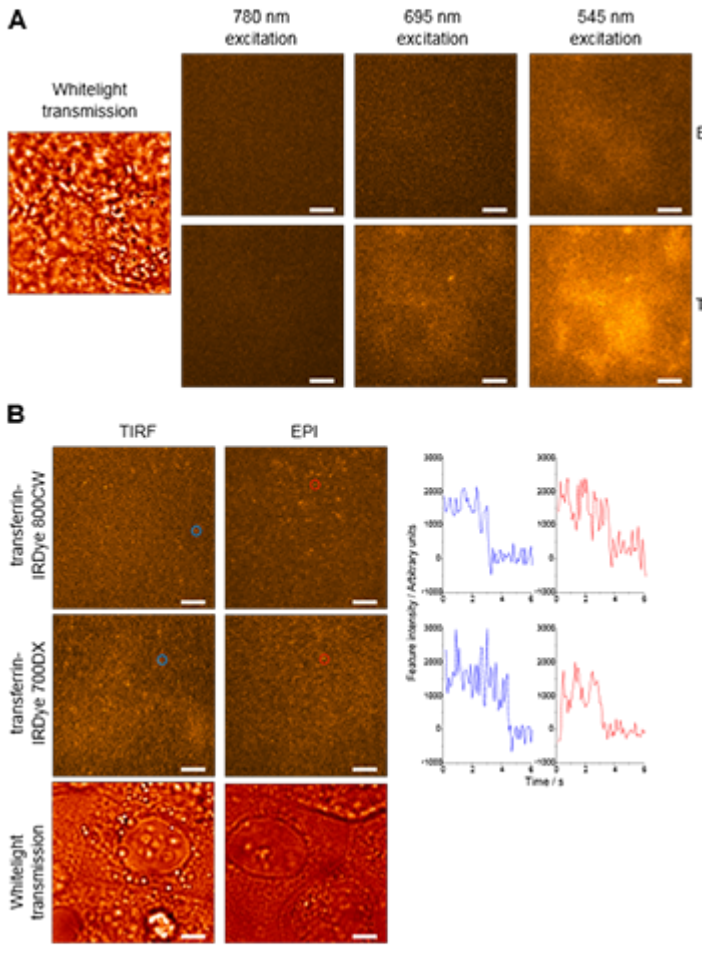

Figure 1. Autofluorescence background and single NIR molecules in MCF-7 cells. A. Images of a single area of unlabelled MCF-7 cells independently illuminated with broadband 780 nm, 695 nm and 545 nm light demonstrate a decrease in autofluorescence background with increasing excitation wavelength. A whitelight transmission image indicates the position of cells within the field of view. B. Images of single molecules of transferrin-IRDye 700DX and transferrin-IRDye 800CW on MCF-7 cells simultaneously illuminated with broadband 780 nm and 695 nm light. Typical intensity vs. time traces, of the molecules highlighted by red and blue circles, are shown to the right.

Andor’s solution for multi-wavelength imaging of single molecules

To measure the weak signals from single molecules it is very important to choose a highly sensitive detector. Andor’s range of iXon Ultra EMCCD’s are the ideal detector for single molecule imaging. They exhibit the ability multiply the photoelectron signal on the camera without having to increase exposure times or illumination intensity, therefore maintaining a photostable sample. The iXon range of EMCCD cameras can achieve very high frame rates in combination with extremely low read noise, again making them highly sensitive for single molecule detection. In order to capture multiple wavelengths simultaneously, TuCam can be used to separate the wavelengths onto two iXon EMCCD cameras. This is what Stephen Webb’s research team did when testing the properties of multiple NIR dyes for single molecule detection as outlined above.