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Controlled Light Exposure Microscopy (CLEM):

improved cell viability during confocal microscopy

Abstract:

Live cell microscopy is used widely to observe cellular targets labelled with a fluorescent peptide. Excitation of the probe, however, especially with laser light, can have a negative impact on cell survival. Both photobleaching and phototoxicity compromise fluorescence time-lapse recordings, especially in confocal microscopy. Controlled Light Exposure Microscopy (CLEM) reduces exposure to potentially damaging excitation light. Reduced photobleaching and increased cell survival were observed in BY-2 tobacco cells and HeLa cells imaged with a Nikon C1 confocal system configured with CLEM compared with a Nikon C1 confocal system without CLEM.

Introduction:

The ability to observe dynamic events in living cells is one of the greatest challenges in biological research. Fluorescent probes, such as green fluorescent protein (GFP) and its derivatives, have helped researchers view specific cell targets and track protein localisation and distribution in living cells. The variety of probes now available (Miyawaki, 2004) enables multi labelling studies in living cells and allows researchers to study interactions between proteins tagged with different fluorescent proteins over time. However, problems associated with light (especially laser light) bleaching of fluorescent probes and phototoxicity (which can lead to cell death) can seriously compromise time-lapse experiments. A new method known as Controlled Light Exposure Microscopy (CLEM) [E Manders; 2006] regulates laser illumination so that photobleaching is reduced and cell survival increased. This application note describes the use of CLEM in association with a Nikon C1 confocal system to image BY-2 tobacco cells and HeLa cells.

Methods:

In a conventional laser scanning confocal microscope system every pixel is illuminated individually by laser light (typically in the micro-second range) and fluorescence emission is detected simultaneously. A confocal image consists of many of these individual pixels and typically displays high (bright) and low (dim) intensity pixels. In general, each pixel is acquired with the same laser power and detector gain (sensitivity) setting. In contrast, when CLEM is used exposure to laser light is determined on a per pixel basis (i.e. when and where required). Excitation light is reduced using two strategies:

1. The first is based on the principle that if there is no signal, then no illumination is required (for example, the background).
2. The second detects whether there is sufficient signal to acquire an image. If so, illumination can be stopped.

Figure 1A shows a schematic overview of the CLEM principle. In "non-CLEM microscopy" an object is uniformly illuminated. Consequently, the detected image is a direct representation of the object. In "CLEM" microscopy the non-uniform illumination is controlled by the detection signal via a feedback system. Combining the illumination image and the detection image forms the final image. Figure 1B shows CLEM configured with the Nikon C1 confocal system. CLEM electronics use the pixel-clock and the detector signal as inputs. An acousto optical modulator (AOM) is used as a fast shutter (the modulation of a solid-state (diode) laser may also be used).

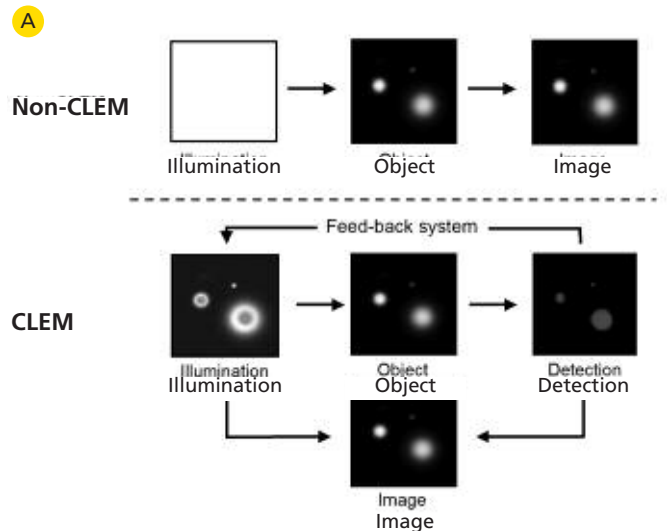
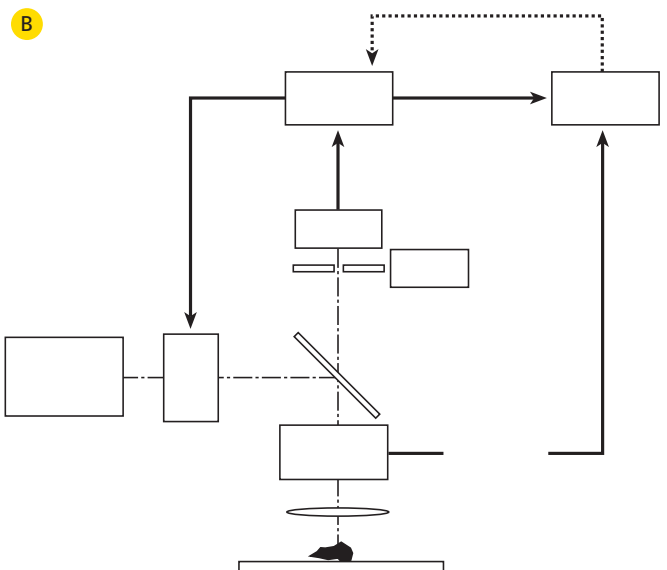


Figure 1.

A) Comparison of non-CLEM and CLEM microscopy.
B) Schematic overview of the C1 system equipped with the CLEM module.



Three investigations were carried out:

1. CLEM was used firstly to image autofluorescence in pollen grains. This was to ensure that there were no differences in the morphology of cells when imaged with and without CLEM.
2. To test the effect of CLEM on photobleaching in living cells, BY-2 tobacco cells expressing GFP-MAP4 associated with microtubules were used in a time-lapse experiment. Identical settings were used for the time-lapse experiment (a 3D acquisition in time) in the presence and absence of CLEM.
3. A reduction in laser excitation should not only reduce bleaching, but should also have a positive effect on cell survival. To test this, HeLa cells expressing GFP tagged histone-2B were plated on a Petri-dish and multiple areas on the dish were imaged in a multi-dimensional time-lapse experiment in the presence or absence of CLEM. Both transmitted light and (GFP) fluorescence images were acquired to obtain optimal morphological information on the cells.

Results:

Figure 2 demonstrates that the morphology of the pollen grain is similar with both non-CLEM and CLEM imaging. However, it can be clearly noted that the light exposure dose of the sample in the absence of CLEM is much higher than in the presence of CLEM. Preliminary calculations (computer simulations) reveal that the phototoxicity is reduced by a factor of six when using CLEM (Hoebe et al, 2006.).

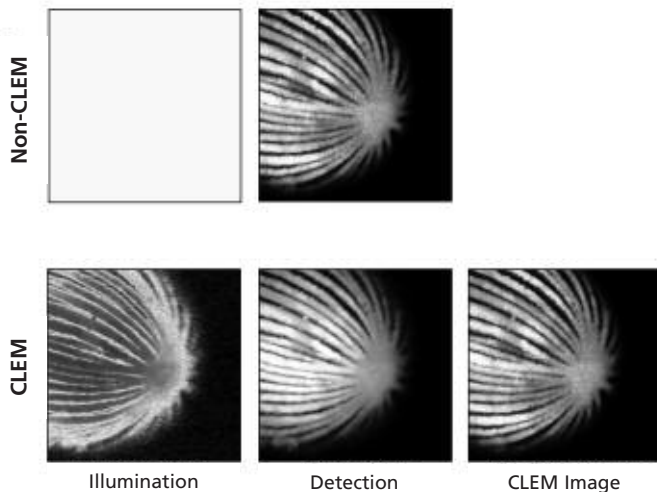


Figure 2. Confocal image of auto-fluorescence of pollen grain in the absence and presence of CLEM. Top panel shows the illumination image (left) and confocal image (right) in the absence of CLEM. Bottom panel shows the illumination image (left), detected image (middle) and CLEM image (right).

In BY-2 tobacco cells expressing GFP-MAP4 associated with microtubules, the GFP-MAP4 expressed by the BY-2 cells is bleached to half of its original fluorescence intensity (arbitrary units) after approximately five 3D scans in the absence of CLEM (Figure 3A). In contrast, in the presence of CLEM, the GFP-MAP4 fluorescence intensity was reduced by 50% after twenty five 3D scans (figure 3B).

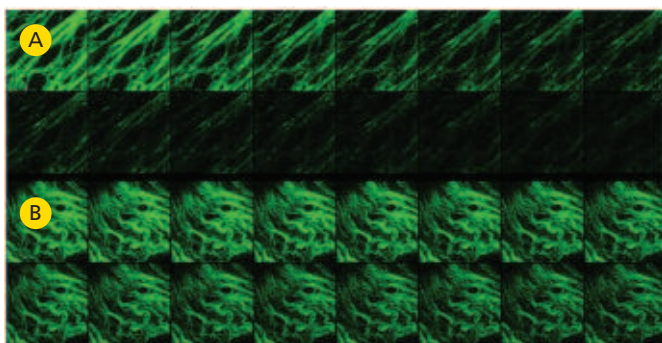


Figure 3. (A) Time-lapse acquisition (XYZ in time) of BY-2 tobacco cells expressing GFP-MAP4 associated with microtubules. The XY image at the middle of the image stack is presented in time. The sequential images were acquired in the absence of CLEM. (B) Time-lapse acquisition (XYZ in time) of BY-2 tobacco cells expressing GFP-MAP4 associated with microtubules. The XY image at the middle of the image stack is presented in time. The sequential images were acquired in the presence of CLEM.

Figure 4 shows a time-lapse acquisition of HeLa cells expressing GFP tagged histone-2B in the presence or absence of CLEM. The intensity levels of the GFP tagged histone-2B remain approximately equal in both cases. In the absence of CLEM, the HeLa cells reveal membrane blebbing at an early stage followed by cell death (top panel). In the presence of CLEM, the cell survival is greatly prolonged (bottom panel).

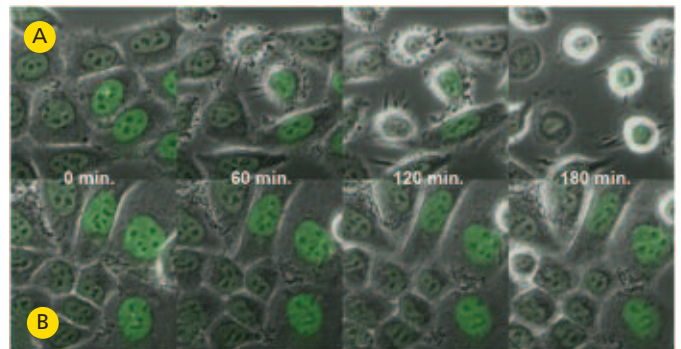
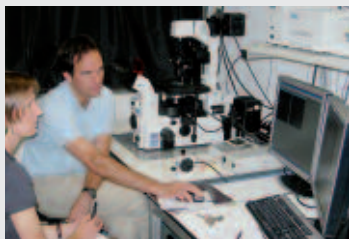


Figure 4. Time-lapse acquisition of HeLa cells expressing GFP tagged histone-2B. The transmitted light and fluorescence images were simultaneously acquired in the absence (A) or presence (B) of CLEM.

CLEM directly affects the pixel-by-pixel laser excitation light dose. While CLEM was created for qualitative live cell imaging, it may have an impact on quantitative studies. The observation that CLEM reduces photobleaching and phototoxicity means that results are less likely to be affected by artefacts related to the processes of cell death. Since the illumination image, detection image and CLEM image are all available, it should be possible to completely correct the CLEM image for accurate quantitative imaging. The increased dynamic range may also offer additional benefits. Since bright pixels are only illuminated for a limited time, it should be possible to extrapolate the detected fluorescence emission intensity. This may contribute to greatly increased dynamic range resulting in an additional advantage for CLEM in addition to reduced photobleaching and improved cell viability.

Conclusion:

Florescent protein expressing cells are sensitive to photobleaching and phototoxicity. In live cell imaging, it is important to minimise these effects to prolong cell survival especially for time-lapse studies. The use of CLEM in a confocal microscope system can reduce the effects of both photobleaching and phototoxicity and is an essential tool for live cell imaging studies.



Maarten Balzar at one of the imaging workstations with Ulrike Engel, Scientific Director of the Nikon Heidelberg Imaging centre.

Authors Background

Maarten Balzar is an application manager for Nikon Instruments Europe BV and is responsible for (biological) research applications such as TIRF, confocal, and advanced fluorescence microscopy. Maarten joined Nikon in 1999 after completing his PhD at the Department of Pathology, Leiden University Medical Centre (LUMC), the Netherlands. His current activities are focussed on new product developments for the (confocal) microscopy product line.

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R. Hoebe, C. van Oven, E. Manders (2006), Focus on microscopy; http://www.focusonmicroscopy.org/2006/PDF/061_Hoebe.pdf.

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■ C1-CLEM reduces photobleaching

■ C1-CLEM minimises phototoxicity

■ C1-CLEM increases cell viability

■ C1-CLEM is a key tool for confocal live cell imaging

For more information on CLEM go to:

www.nikoninstruments.com/clem



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