**Controlled Light Exposure Microscopy (CLEM)**

**New Imaging Approach Reduces Photobleaching and Phototoxicity**

Photobleaching and phototoxicity are often the Achilles’ heels of fluorescence live-cell imaging. Controlled Light Exposure Microscopy (CLEM) is a novel, simple imaging approach that reduces photobleaching and phototoxicity 2 to 10-fold without compromising image quality. The basic concept of CLEM is that light is only used where it is needed by spatial control (pixel by pixel) of the light exposure time. Experiments show that CLEM results in a 2 to 10-fold reduction of photobleaching and phototoxicity. As a bonus, CLEM increases the dynamic range of the fluorescence intensity at least by a factor of two.

**Introduction**

High-end microscopy, especially live-cell fluorescence microscopy, is often a matter of finding the optimal compromise between several imaging properties. When a fluorescence signal is weak (low signal-to-noise ratio (S/N)), one could increase the laser power, but that introduces stronger photobleaching and phototoxicity. In the similar way S/N is influenced by the size of the pinhole, which is directly coupled to the resolving power, pixel size, number of pixels and consequently to light exposure time- and thus photobleaching and phototoxicity. In brief: finding the right settings of the confocal microscope for a specific sample is a matter of finding the balance between S/N and phototoxicity and photo-bleaching (fig. 1). One thing is clear: you can't have it all!

CLEM is a new technology that breaks this balance. Controlled Light Exposure Microscopy (CLEM) enhances cell viability without deteriorating image quality. The basic concept of CLEM is that excitation light is used only where it is needed by spatial control (pixel by pixel) of the light exposure time [1-3].

**The Concept of CLEM**

To explain this novel concept of imaging, we first have a better look at the S/N ratio of a typical conventional (non-CLEM) confocal image as depicted in figure 2a. The image shows an optical section through three objects with different sizes and
intensities and, consequently, each with a different local S/N. This is due to the fact that S/N is directly related to the intensity, since quantum noise (or Poisson noise) is generally the main source of noise in fluorescence (confocal) microscopy.

In fact, the S/N is different for every individual pixel in non-CLEM due to non-uniform distribution of fluorophores and uniform illumination of the sample. In non-CLEM imaging, the local excitation light dose is constant throughout the field of view and the over-all intensity is adjusted to the weakest objects. Consequently, bright foreground (red in fig. 2b) has a S/N that is far too good. In the background (blue in fig. 2b) there is no need for a good S/N since there is no signal in the background. In both areas, background and bright foreground, the local excitation dose is higher than needed. Therefore, a large portion of the excitation light dose in non-CLEM does not contribute to improvement of image quality (S/N) and thus leads to unnecessary photobleaching and phototoxicity.

CLEM is a fundamentally different concept of imaging based on non-uniform illumination of the field of view. CLEM regulates the number of photons emitted by the specimen for every individual pixel by controlling the excitation light exposure time. In brightly fluorescent pixels (red in fig. 2b) CLEM reduces the exposure time since there the S/N is more than sufficient. In background pixels (blue in fig. 2b) the light exposure time is also reduced since it is pointless to obtain a good S/N in areas without any signal. Weak foreground pixels (grey in fig. 2b) are illuminated with the regular exposure time.

It is important to realize that photo-bleaching and phototoxicity are generated not only in the focal plane (in-focus), but also in the entire light cone above and below the focal plane (out-of-focus). This means that, when light exposure time is reduced in-focus, photobleaching and phototoxicity will be reduced both in-focus and out-of-focus. Therefore, the actual reduction of photobleaching and phototoxicity depends on the 3D distribution of fluorophores in the sample when CLEM is applied.
**Confocal CLEM**

CLEM has been implemented in a standard scanning confocal microscope (fig. 3). Two alterations of the conventional set-up were sufficient to install CLEM. A fast shutter (acoustic-optical modulator) is placed in the laser beam in order to switch on and off the illumination light within the pixel dwell time. An electronic circuit, which functions as a fast feedback system, controls the shutter depending on signals from the detector and calculates the signal for the formation of the image. With this relatively small adaptation, a CLEM image is produced with the same scanning speed as a non-CLEM image. In practice, a CLEM image has the same image quality as compared with a non-CLEM image. Only detailed inspection shows that the CLEM has a decreased S/N in background and bright foreground.

**Reduced Photobleaching**

CLEM allows confocal imaging of bleach-sensitive objects. For both fixed and living cells photobleaching is reduced 2- to 10-fold. Figures 4a and b show a series of single optical sections from 3D images of fixed tobacco plant BY-2 cells expressing a microtubule reporter GFP-MAP4 [3] (see the Nature Biotechnology website (www.nature.com/nbt) for a video). Analysis of the bleaching curves (fig. 4c) shows that the bleach-rate in CLEM is 7-fold slower than bleach rate in non-CLEM.

**Reduced Phototoxicity**

CLEM allows prolonged imaging of photo-sensitive fluorescently labelled cells due to increased cell viability under CLEM imaging conditions. Figure 5 shows living HeLa cells expressing the H2B-GFP fusion protein during time-lapse imaging with non-CLEM and CLEM [3] (see the Nature Biotechnology website (www.nature.com/nbt) for a video). Cells imaged with non-CLEM show blebbing of cell membranes for the first time after approximately half an hour. After approximately three hours, most cells are rounded-up and some are apoptotic. Under CLEM imaging conditions, blebbing occurs only after approximately three hours. Apparently, reduction of the excitation light dose by CLEM allows prolonged live-cell imaging (in this case approximately six times longer) without loss of image quality (S/N).

**Increased Dynamic Range**

Clipping of a fluorescence signal is a well-known effect when the high voltage (gain) of a photomultiplier tube (PMT) is set too high. The signal cannot reach a higher value than the number of bits of the digitized signal allows. Often this effect is hard to prevent when in the same image some objects are very bright and some are very
faint. When CLEM is applied, clipping of a signal is a phenomenon that does not exist anymore since the light exposure time is reduced before the intensity reached the highest value. The final image intensity equals the ratio of the measured intensity and the exposure time. In conclusion, the dynamic range expressed as bit-depth is at least doubled by using CLEM. In practice, when after analogue-digital conversion the measured fluorescence signal and the exposure time are expressed by 8-bits, the calculated fluorescence intensity can be expressed by 16 bits.

Conclusion

CLEM is a novel approach to address two serious restrictions in fluorescence microscopy: photobleaching and phototoxicity. As a bonus, CLEM improves the dynamic range of the fluorescence intensity. The benefit of CLEM with respect to photobleaching can be exploited in most applications of fluorescence, both in fixed and live-cells. Reduction of phototoxicity by CLEM is important for most applications in live-cell imaging, especially for the study of cells over longer periods. Moreover, CLEM increases the number of good-quality images that can be captured by exploiting every detected photon in the most efficient way.

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References:


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original work on www.nature.com/nbt.

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