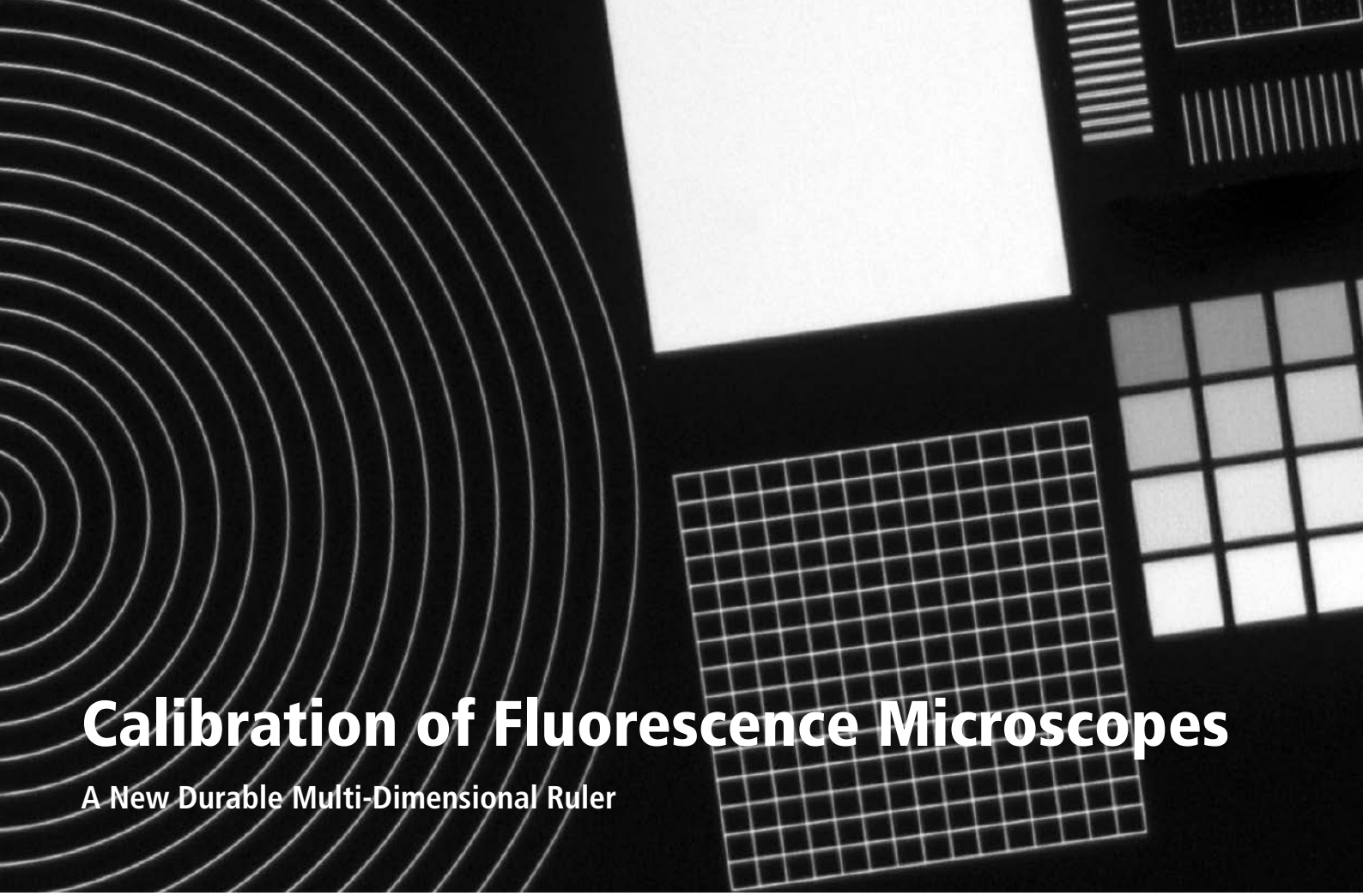

Calibration of Fluorescence Microscopes

A New Durable Multi-Dimensional Ruler

We propose a new durable multi-dimensional ruler for the calibration and the alignment of fluorescence microscopes (wide-field, confocal, multiphoton), thanks to non-photo-bleaching fluorescent sub-micrometer patterns embedded in glass in three dimensions. This new and robust tool enables the measurement of stage repositioning, detector's features, illumination homogeneity, both lateral and axial resolutions as well as the spectral shape, intensity and lifetime response of the system.

Keywords

Fluorescence, Microscopy, Calibration, Multidimensional, Ruler

Context

Fluorescence microscopes have become ubiquitous in many laboratories in biology, medicine, and materials science. For the past few years, the need for pursuing a quality approach has been arising, mainly driven by the search for guaranteed performances and comparable quantified results. However, this need faces a limitation, which is that fluorescence microscopes are sold as "imaging tools" and not as "measurement tools". This restriction is due to (i) the complexity of these devices, which does not allow their different components to be calibrated altogether, (ii) a lack of reference materials in this field. For these reasons, the performances of fluorescence microscopes are not monitored as often as their use would require. This is even emphasized by microscope manufacturers such as Carl Zeiss Microscopy themselves [1]: "In the 20 years or more since introduction of laser scanning confocal microscopes, it has not been feasible to assess how these systems compare. No one can assert with confidence that instrument A in use in 1990 has better or worse sensitivity than instrument B operating in 2006. There is, therefore, a paramount need for standardized test samples and procedures for their use."

According to the broad community of fluorescence microscopists, the ideal calibration tool for fluorescence microscopy would be able to characterize (non-exhaustively) [2]: The point-spread function, the spatial resolution (x, y and z), the spectral irradiance reaching the sample, the illumination homogeneity, the field flatness, z-distances, the spectral resolution, the day-to-day and long-term instrument performances, as well as the detector's range of linearity, dynamic and limit of detection. It should also not photo-bleach, be reliable and durable, and easy to use and handle.

Argolight, a spin-off from the University of Bordeaux, specializes in processes for the multi-scale (from sub-µm to cm) marking and engraving of stable fluorescent objects in three dimensions (3D) inside transparent materials. Such non-photo-bleachable patterns in glass ideally enable the calibration of fluorescence microscopes. In this paper, we present a few aspects of a fluorescent multi-dimensional (space, intensity, spectrum, lifetime) ruler adapted for the calibration and the alignment of wide-field, confocal, and multiphoton microscopes or their components. A potential application on what a fluorescence microscope calibrated with this tool could allow in fluorescence *in situ* hybridization (FISH) is also discussed.

Calibration Slide

Figure 1A shows a typical calibration slide, consisting of a glass substrate on

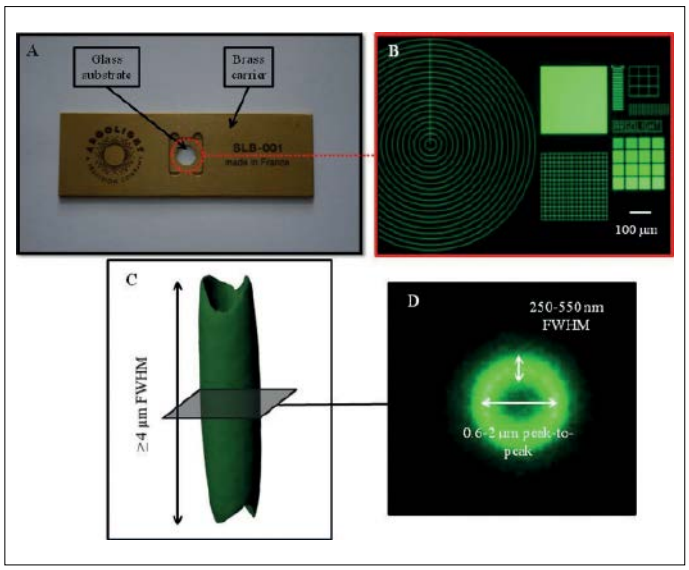

Fig. 1: (A) Typical calibration slide with fluorescent patterns engraved inside the glass, (B) wide-field image of these fluorescent patterns (λ_{exc} = 365 nm, λ_{em} = 445 ± 25 nm, 5 × / 0.15), (C) reconstructed confocal fluorescence image (λ_{exc} = 405 nm, λ_{em} = 420-700 nm, 40×/1.25) of the pipe-shaped elementary pattern, (D) cross-section of the pipe.

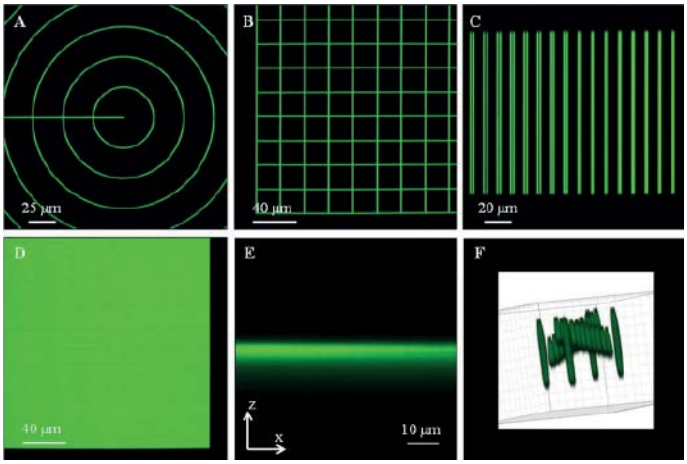

Fig. 2: Confocal fluorescence images (λ_{exc} = 405 nm, λ_{em} = 420-700 nm, 63 × / 1.4) of different patterns having different functions: (A) a circular target, (B) a squared grid, (C) lines separated from gradually increased distances, (D) a homogeneous filled square, (E) xz view of the same square, (F) reconstructed "crossing stairs".

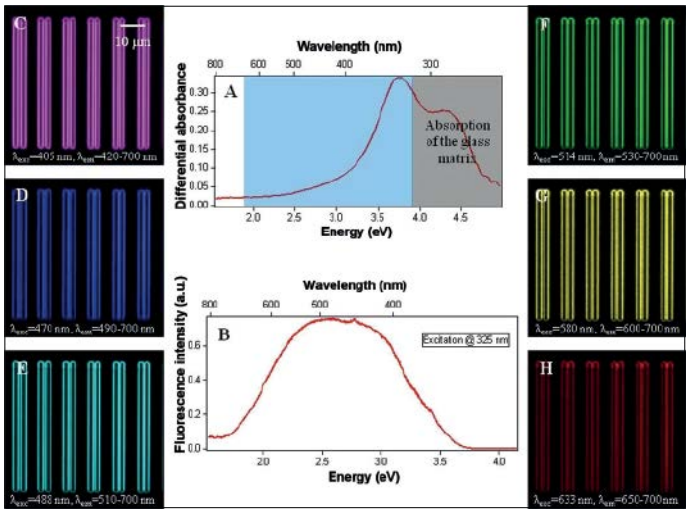

Fig. 3: (A) Typical absorbance and (B) true emission (λ_{exc} = 325 nm) spectra of the engraved patterns, (C to H) confocal fluorescence images (40 × / 1.25) of the same lines taken with different excitation and emission wavelengths.

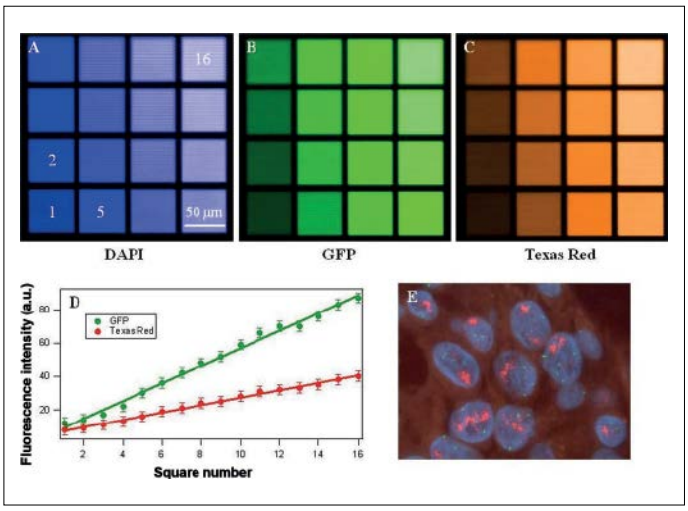

Fig. 4: Wide-field images (20 × / 0.8) of 16 square patterns having different fluorescence intensities acquired with excitation and emission filter sets corresponding to common fluorescent stains [(A) DAPI (λ_{exc} = 365 nm, λ_{em} = 445 ± 25 nm), (B) GFP (λ_{exc} = 470 ± 20 nm, λ_{em} = 525 ± 25 nm), (C) Texas Red (λ_{exc} = 550 ± 12 nm, λ_{em} = 605 ± 35 nm)], (D) fluorescence intensity levels of these squares for GFP and Texas Red filter sets, (E) wide-field fluorescence image of breast carcinoma cells' nuclei, HER2 proteins and CEP17 chromosome centromeres labeled with DAPI, Texas Red and GFP, respectively (from [3]).

a brass carrier, featuring the same dimensions as a standard microscope slide. Various fluorescent patterns are embedded inside the glass (fig. 1B). Each pattern is intended and designed to respond to a particular calibration issue. The elementary pattern is a hollow cylinder (pipe shape) with a FWHM (Full Width at Half Maximum) length that can be varied from 4 to tens µm, a peak-to-peak diameter of 0.6 to 2 µm, and a FWHM wall thickness of 250 to 550 nm (figs. 1C & 1D). From this elementary pattern, any more complex linear or circular geometrical 3D shape can be achieved.

Figure 2 displays a series of patterns that have different functions. The circular geometry of the target shown in figure 2A allows checking if the scanning rotation in a confocal microscope does not modify or move the image. This structured pattern permits also to verify if the reconstruction of different images into a mosaic is accurate. Associated to a dedicated software, stage repositioning can also be measured. The grid shown in figure 2B allows checking the presence of distortion and, like the target, permits to verify the accuracy of image reconstruction. Pairs of lines, which spacing is gradually increased from 100 nm, are intended to measure the lateral resolution (fig. 2C). The value of the spacing between two lines that can be spatially resolved gives the lateral resolution. The filled pattern in figure 2D enables the measurement of the illumination homogeneity, as well as the field flatness (fig. 2E). The pattern in figure 2F that con-

tains two "crossing stairs" with features at different depths allows the verification of the optical sectioning of a confocal microscope and the observation of any drift of the stages once the image is reconstructed.

Spectral Features

Spectrum

An incontrovertible advantage of this slide concerns its extremely broad excitation and emission spectra. The excitation spectrum ranges from 325 to 650 nm (fig. 3A) while the emission wavelengths are in the visible to NIR (near infrared) range when excited by ultraviolet (UV) up to blue radiation (fig. 3B). More importantly, it is a continuum, enabling any spectral calibration of a fluorescence microscope, and in particular any chromatic aberration investigations. Figure 3C to 3H shows the same patterns imaged with different excitation wavelengths and different emission spectral ranges.

Lifetime

Lifetime measurements have been performed (not shown here). They reported a fast decay component with a lifetime of approximately 1.2 ns, compatible with FLIM (Fluorescence Lifetime Imaging Microscopy) studies. Other decay components are also present, but their measurement could not be performed with good accuracy.

Intensity

Figure 4A to 4C shows 16 squares emitting different fluorescence intensities, for different excitation wavelengths and emission spectral ranges corresponding to three extensively used fluorescent stains: DAPI, GFP and Texas Red. The measured global fluorescence intensity of each square is plotted versus the square number in figure 4D. For each excitation and emission spectral ranges, one can see that 16 fluorescence intensity levels can be well discriminated, and that they follow a linear evolution, with a different slope. This pattern provides therefore a "fluorescence intensity ruler", which can be used to scale any fluorescence intensity measurement. This is particularly useful for the investigation of the dynamic as well as the linearity range of the detectors' response. It might also open the road towards quantitative analysis, as it will be discussed in the next section.

Potential Application in FISH

Fluorescence *in situ* hybridization (FISH) is a well-established technique used for therapeutic diagnosis of cancers. For example, in breast cancer, the HER-2 proteins and CP17 chromosome centromeres as well as the nuclei of carcinoma cells are targeted by Texas Red, GFP and DAPI fluorescent biomarkers, respectively [3]. An image performed with a fluorescence microscope shows spatially distributed red (HER-2) and green (CP17) aggregates (fig. 4E). A pathologist then analyzes the image, by counting the number of HER-2 and CP17 signals, and determines if the HER-2 protein is over-expressed. However, the fluorescence intensity of the HER-2 and CP17 signals in this kind of images is not exploited. A "fluorescence intensity ruler", like the one shown in figures 4A to 4D, could probably provide an additional dimension for a finer or more complete interpretation.

Conclusion

Argolight's non photo-bleaching fluorescent sub-micrometer patterns are suited to fulfill the requirements of a reference tool for the calibration and alignment of fluorescence microscopes. They provide sub-resolution feature sizes of 250 nm in xy- and 4 µm in z-direction, respectively, remarkable spectral features (broad excitation and emission spectra, short lifetime, different intensity levels) and long-term stability. In addition to knowing and monitoring the performances of fluorescence microscopes, this multi-dimensional ruler could also open new possibilities in the field of fluorescence *in situ* hybridization based clinical diagnosis, and in extension probably in any field where a fluorescence intensity scale is required.

Acknowledgements

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Contact
Dr. Arnaud Royon
Dr. Gautier Papon
Argolight SA
Talence, France
a.royon@argolight.com
www.argolight.com