Simple Tool for the Standardisation of Confocal Spectral Imaging Systems

In confocal fluorescence microscopy, comparability of measurements across instruments, laboratories, and over time is still limited. To rule out instrumentation as a major source of variability, reliable standards in combination with tested protocols for instrument characterisation and performance validation are required. A first step is a simple calibration tool for the standardisation of the spectral characteristics of confocal spectral imaging systems.

Introduction

Confocal laser scanning fluorescence microscopy (CLSM) has been developed into one of the most powerful methods of analysis in the life sciences. It has outgrown from being a simple imaging technique and matured to a state where quantification is desired and many applications in strongly regulated areas like e.g. medical diagnostics are at reach. This is in striking contrast to the lack of standardised procedures for instrument characterisation and performance of typical measurements, especially as major sources of error and deviation in microscopic data are often linked to complex instrumentation.

Need for Standards

Improved quality assurance in CLSM relies on easy-to-operate standards and reference materials in combination with widely accepted protocols for the determination of microscope parameters like e.g. spectral irradiance at the sample, field flatness, homogeneity of illumination, spectral and spatial resolution, and spectral sensitivity of the emission channel as well as their time-dependent fluctuations [1, 2]. The latter is closely linked to the increasing importance of long-term studies in biology and medicine. Suited standards must be well characterised with respect to their calibration-relevant properties, easy to align with high reproducibility, applicable under typical measurement conditions, and of sufficient long-term stability or excellent reproducibility.

Spectral Characteristics of Laser Scanning Microscopes (LSM)

Similarly to other fluorescence techniques, CLSM yields signals that contain sample- and instrument-specific contributions. The former are desired, the latter hamper the comparability and combination of data obtained on different instruments, introduce a time-dependence due to aging of optical and optoelectronic components in microscopes, and render quantification difficult. Instrument-specific effects are determined by the wavelength-dependent spectral irradiance at the sample position, the wavelength-dependent light collection properties and the aberration correction of the microscope as well as by the wavelength-dependent spectral sensitivity of the emission detection system [2]. Reliable and comparable microscopic data require control, determination, and consideration of these effects.

Spectral Standards

Spectral fluorescence standards like dyes C-E with known corrected, i.e., instrument-independent broad and unstructured emission spectra \(I_c(\lambda_{em})\) that present chemical equivalents of calibration lamps, are simple tools for the determination of the relative spectral responsivity or sensitivity \(s(\lambda_{em})\) of the emission channel of fluorescence instruments under application-relevant conditions [2]. This quantity equals the quotient of the corrected spectra divided by the uncorrected, i.e., instrument-dependent spectra \(I_u(\lambda_{em})\) measured with the instrument to be calibrated, see Fig. 1. Dyes C-E and their short-wavelength analogues dyes A and B will be certified by the Federal Institute for Materials Research and Testing (BAM) in fall 2005. These dyes have been tested for thermal and photochemical stability, dependence of emission spectra on excitation wavelength, and fluorescence anisotropy.

Fig. 1: Relative inverse spectral responsivity of two LSMs determined with BAM spectral fluorescence standard dyes C-E.
Adaptation to Confocal Fluorescence Microscopy (CLSM)

As a first step towards standardised instrument characterisation in confocal fluorescence microscopy, we designed a calibration procedure based upon a slide with integrated micro-channels, shown in Fig. 2 (ibidi GmbH Integrated BioDiagnost), filled with dyes C-E for measurement of fluorescence at defined z-positions. Its use for the determination of the (inverse) relative spectral sensitivity of LSsMs is displayed in Fig. 1. Aside from linking fluorescence measurements to the spectral radiance scale, this tool also enables validation of instrument performance and long-term stability via regular measurement of the spectral characteristics of LSsMs at constant instrument settings like e.g. excitation wavelength, beam splitter configuration, PMT voltage, and alignment of the emission pin-hole. Currently, we test its applicability for the determination of the range of linearity of emission detection systems and for control of spectral unmixing algorithms offered as a solution for the separation of overlapping dye emission spectra by the majority of manufacturers of LSsMs.

References


Dr Katrin Hoffmann
Dr Ute Resch-Genger
Federal Institute for Materials Research and Testing (BAM)
Working Group Optical Spectroscopy
Richard-Willstätter-Str. 11 - 12489 Berlin, Germany
Tel. +49 30 8104 1134 · ute.resch@bam.de

Dr Roland Nitschke
Albert-Ludwigs-Universität
Life Imaging Center, Institute of Biology I
Developmental Biology, Hauptstr.1
79104 Freiburg, Germany
Tel. +49 761 2032934 - Fax +49 761 2032941
roland.nitschke@biologie.uni-freiburg.de