TauSense: a fluorescence lifetime-based tool set for everyday imaging

The TauSense technology from Leica Microsystems is a new, straightforward way to generate images using lifetime-based information. Measuring changes in the fluorescence arrival times gives an extra layer of information for understanding the functions of molecules within the cellular environment, increasing image quality, and expanding the number of probes that can be visualized in a specimen. This technology enables the acquisition of lifetime-based information with smaller data size and computational load compared to fluorescence lifetime imaging (FLIM). Moreover, the implementation of different tools (TauConstrast, TauGating, TauScan and TauSeparation) allows exploration of this extra dimension of information at different levels.

Fluorescence microscopy allows researchers to gain unprecedented insights into cellular mechanisms. An intrinsic phenomenon of the fluorescence process is the characteristic time between the excitation of the molecule and the photon emission (Fig. 1a). This time is known as fluorescence lifetime and corresponds to the time that the molecule stays in the excited state (S_1) before going back to the ground state (S_0). It is typically in the sub-nanosecond-to-nanosecond range. In this last step a photon is generated, as portrayed in the Jablonski-Perrin diagram (Fig. 1a)[1]. The average fluorescence lifetime is a characteristic value for each fluorophore, but it can vary if there are changes in the fluorophore's close vicinity (less than 10 nm)[2]. The changes in the fluorophore microenvironment that translate into changes in fluorescence lifetime make it a powerful analysis tool in life sciences. These changes have been used, for example, in the design of fluorescence-lifetime-based biosensors[3].

It is possible to probe lifetime-based changes by measuring the photon arrival times[4]. A straightforward way of determining arrival times is by measuring the difference between the time when a photon is detected and the time of the excitation laser pulse (Fig. 1a). The zero value of the measurement can be calibrated using the reflection signal from the excitation laser reaching each detector. Having access to the arrival times can reveal information complementary to the fluorescence intensity, which allows characterization of a specimen or a process of interest. As an example (Fig. 1b), lifetime-based information in images of an *Arabidopsis thaliana* root–hypocotyl junction expressing an actin fluorescent marker[5] and labeled with propidium iodide shows three distinct structures—actin network, cell wall and chloroplasts—that are difficult to identify when only the intensity information is available.

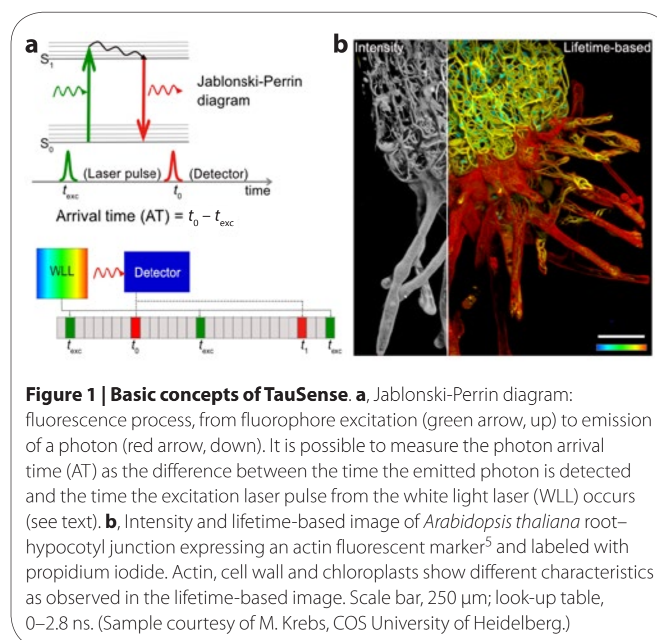

Figure 1 | Basic concepts of TauSense. **a**, Jablonski-Perrin diagram: fluorescence process, from fluorophore excitation (green arrow, up) to emission of a photon (red arrow, down). It is possible to measure the photon arrival time (AT) as the difference between the time the emitted photon is detected and the time the excitation laser pulse from the white light laser (WLL) occurs (see text). **b**, Intensity and lifetime-based image of *Arabidopsis thaliana* root–hypocotyl junction expressing an actin fluorescent marker[5] and labeled with propidium iodide. Actin, cell wall and chloroplasts show different characteristics as observed in the lifetime-based image. Scale bar, 250 μm; look-up table, 0–2.8 ns. (Sample courtesy of M. Krebs, COS University of Heidelberg.)

Lifetime-based measurements, carried out on a point-scanning microscope, require a pulsed-laser excitation source; fast, low-noise photon counting detectors; and electronics with appropriate time-resolution capabilities. This is the basic concept underlying the TauSense technology for fluorescence imaging: the use and handling of photon arrival times to generate lifetime-based information for

M. Julia Roberti[1], Laia Ortiz Lopez[1,2,3], Giulia Ossato[1], Irmtraud Steinmetz[1], Petra Haas[1], Frank Hecht[1] and Luis A. J. Alvarez[1*]

[1]Leica Microsystems CMS GmbH, Mannheim, Germany. [2]Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Centre National de la Recherche Scientifique, UMR 7104, and Institut National de la Santé et de la Recherche Médicale, U964, Illkirch, France. [3]Université de Strasbourg, Illkirch, France. *e-mail: luis.alvarez@leica-microsystems.com

different applications. The scan head electronics, supported by a field-programmable gate array, sort the detected photons into digitally preset gates depending on their arrival times. This architecture enables TauSense to use the photon time-tagged information only for online calculations—for example, to estimate the average photon arrival time (AAT) values on the fly. The resulting images come from the evaluation of lifetime-based information, but no longer carry the time dimension at the single-photon level. This efficient handling of the data translates into a smaller file size and computational load compared to fluorescence lifetime imaging microscopy (FLIM) methods. As such, TauSense does not aim to replace FLIM methods. The motivation for TauSense is to provide access to a lifetime-based level of information with every point-scanning microscope in a guided way and obtain a result in a straightforward manner. With this in mind, TauSense is a set of tools that enable exploring lifetime-based information at different levels: TauContrast, TauGating, TauScan and TauSeparation.

A first approach to accessing lifetime-based information using TauSense comes from the analysis of photon arrival times for every pixel. The arrival times are handled at the level of the scan head electronics. Taking these together with the pixel information, it is possible to calculate the average photon arrival time per pixel and to generate an image that contains both the intensity (photon counts) and the AAT information for every pixel (Fig. 2a). This approach yields an extra, lifetime-based dimension or contrast that can be used to elucidate more characteristics of a fluorescent signal of biological relevance. This is referred to as TauContrast in TauSense (Fig. 2a). A typical example is the assessment of intracellular pH changes through lifetime-based measurements using suitable fluorescent probes, as shown here for a near infrared membrane stain (Fig. 2b). In addition to staining the plasma membrane, the probe is internalized over time, and the vesicles carrying the probe appear as bright spots in the fluorescence intensity image (Fig. 2b). That is all we can tell about the internalization process using intensity-based information. The corresponding TauContrast image (Fig. 2c) reveals another level of information: the AAT values at the plasma membrane are different than those in the vesicles. These variations in TauContrast correlate with the different environment the dye encounters when transitioning from the plasma membrane first into endosomes and then into lysosomes of different levels of maturation. TauContrast differs within the vesicles and reports on the different pH values present.

Because of the way TauContrast is generated (calibration relative to the timing of the excitation pulse, determined by on-the-fly calculation of the AAT at the scan head electronics), the lifetime-based information is qualitative and can be used in a semi-quantitative way relative to an appropriate control[6]. Like other lifetime-based tools, TauContrast measurements are independent of fluorescence intensities. TauContrast not only allows the monitoring of changes relative to a control sample but is also a precise tool for observing changes within a given sample.

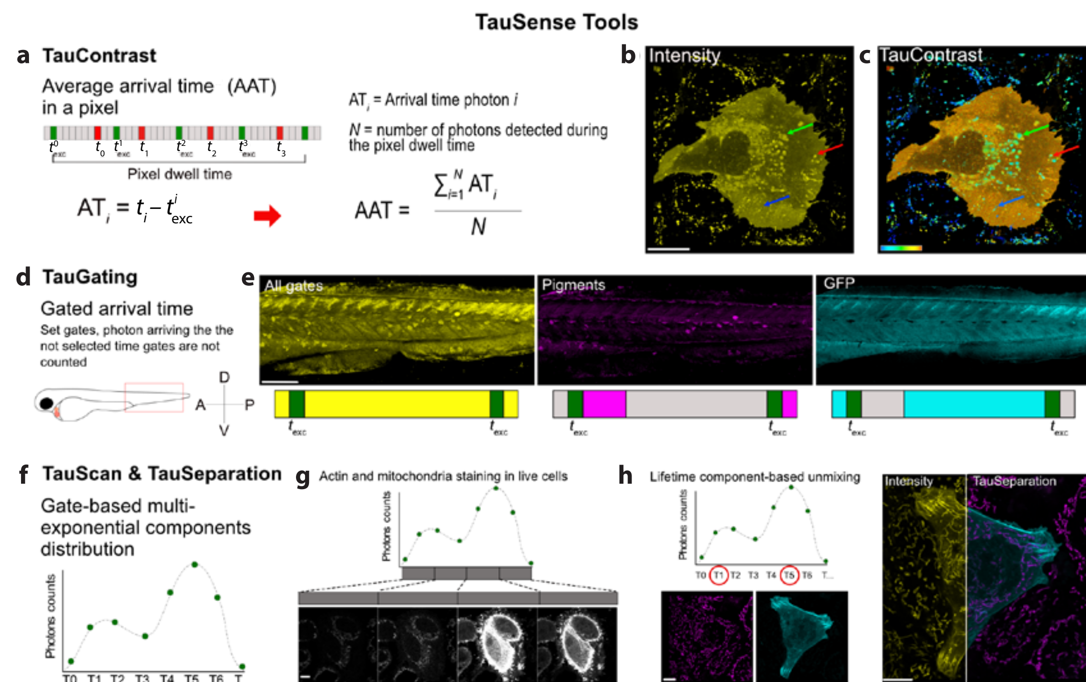

Figure 2 | TauSense tools. a, TauContrast. The contrast in each pixel is given by the average arrival times of the photons detected during the pixel dwell time. **b**,**c**, Mammalian cell labeled with near infrared membrane stain. The arrows indicate vesicles with different pH (red higher, blue lower, green intermediate). **b**, The intensity image, with higher intensity from vesicles. **c**, TauContrast image. Scale bar, 20 µm; look-up table, 0–1.5 ns. Changes in AAT highlight changes in pH in the vesicles during internalization. **d**, TauGating enables splitting photons arriving at different times. **e**, Zebrafish image; photons with short arrival times (pigments) are separated from photons with longer arrival times (GFP signal). Scale bar, 200 µm (see text for details). **f**–**h**, TauScan and TauSeparation. **f**, Schematic of the lifetime components distribution. **g**,**h**, TauScan (**g**) and TauSeparation (**h**) of mammalian cells expressing LifeAct-GFP (manufactured by ibidi GmbH) and labeled with a green mitochondrial stain. Scale bars, 10 µm.

A different use of the arrival times consists of using them to assign photons to 'categories' defined according to certain rules and to generate different populations as a result. The classic example of this approach is gating. TauSense offers this possibility with the TauGating tool. TauGating (Fig. 2d) enables the positioning of multiple digital gates (up to 16) with precise and flexible time definitions. TauGating delivers images that contain the number of photons (intensity) detected in one gate (or a set of gates, if several gates are pooled) during the pixel dwell time. A classic application for TauGating is the isolation of a signal of interest from intrinsic or spurious contributions to fluorescence in a specimen. As an example, we imaged zebrafish of the *4xGTIIC:d2GFP* line[7] still containing their native pigments. The fluorescence signal of interest provides a readout of Yap1/Taz-Tead activity and is used here to visualize the striated muscle of the trunk at 55 hpf (Fig. 2e). Using TauGating, we can extract the signal of interest from the contributions of the endogenous pigments. It is worth noting that we generated not only a gated image containing the relevant signal, but also an image with the 'gated-out' information to ensure the quality and accuracy of the result.

In addition to providing information based on AAT (TauContrast) and splitting signals into gates (TauGating), TauSense opens the door to a detailed characterization of a specimen in terms of the lifetime-based populations of the detected photons. Previous efforts to harness such information from fluorescence signals have described the use of so-called lifetime distributions to assess the potential lifetime components that constitute a given sample[8,9]. To this end, we have developed two TauSense tools, TauScan and TauSeparation, to take full advantage of our ability to characterize the distribution of mean lifetime components (Fig. 2f). Using TauScan, it is possible to scan these distributions and separate them into a predetermined number of temporal windows (Fig. 2g). We use digitally preset gates followed by a multi-exponential components fit to generate an online view (Fig. 2g, top) of the distribution of such lifetime components from the arrival times. The distribution of components enables one to work with the arrival time information in an analogous way to the spectral distribution of a fluorescence signal. Here we obtain the temporal dispersion of the signal, and by positioning the appropriate temporal windows we can split the photon signal according to the arrival time information contained in the photon flux. After such splitting of the signal, the spatial coding of the photons leads to the corresponding images. The images that result from a TauScan experiment are intensity images containing discrete temporal information (the temporal windows as explained above) across the lifetime component distribution. An example of TauScan describes the lifetime-based distributions in live cells expressing LifeAct-GFP (ibidi GmbH)[10] that were labeled with a green mitochondrial stain (Fig. 2g).

The second tool, TauSeparation, uses the lifetime-based distributions for a different application, namely species separation. Although spectral separation in fluorescence imaging has become routine[11] and a powerful tool for distinguishing multiple fluorophores, there are still instances in which fluorophore choices are limited and the necessary spectral differences are difficult to achieve. This can happen, for example, in experiments with animal models in which GFP or similar fluorescent protein reporters are used to track specific mutations and ensure the correct phenotypes. In this the case, the spectral windows of such labels are unavailable for fluorophores with overlapping emission spectra. This result can limit flexibility for probing additional structures or functions. Lifetime-based information can offer a way to tackle such dilemmas. We applied TauSeparation on the cells expressing LifeAct-GFP and stained with the green mitochondrial marker, previously used for TauScan. With TauSeparation, we obtained a well-defined signal from both the mitochondria and the actin filaments in separated intensity images. In TauSeparation, the user decides on the values of the most representative mean lifetime components with the aid of the online diagram. Then TauSeparation selects appropriate temporal windows and fits the lifetime-based information in these windows to generate separated images.

In summary, we present TauSense and show typical applications that benefit from this technology. The TauSense tool set allows one to integrate lifetime-based information and adds freedom for the multiplexing of several probes in everyday fluorescence imaging. TauSense is one of the pillars of the STELLARIS microscopes.

REFERENCES

1. Valeur, B. *Molecular Fluorescence: Principles and Applications. Methods* vol. 8 (2001).
2. Förster, T. Transfer Mechanisms of Electronic Excitation Energy. *Radiat. Res. Suppl.* **2**, 326–339 (1960).
3. Greenwald, E. C., Mehta, S. & Zhang, J. Genetically encoded fluorescent biosensors illuminate the spatiotemporal regulation of signaling networks. *Chem. Rev.* **118**, 11707–11794 (2018).
4. Becker, W. *The bh TCSPC Handbook*. Photon Counting Histograms. 580 (Becker & Hickl, 2017).
5. Era, A. et al. Application of lifeact reveals F-actin dynamics in *Arabidopsis thaliana* and the liverwort, *Marchantia polymorpha. Plant Cell Physiol.* **50**, (2009).
6. Jerome, W. G. J. & Price, R. L., eds. *Basic Confocal Microscopy* (Springer International, 2018); https://doi.org/10.1007/978-3-319-97454-5
7. Miesfeld, J. B. & Link, B. A. Establishment of transgenic lines to monitor and manipulate Yap/Taz-Tead activity in zebrafish reveals both evolutionarily conserved and divergent functions of the Hippo pathway. *Mech. Dev.* **133**, 177–188 (2014).
8. James, D. R. & Ware, W. R. Recovery of underlying distributions of lifetimes from fluorescence decay data. *Chem. Phys. Lett.* **126**, 7–11 (1986).
9. Mérola, F., Rigler, R., Holmgren, A. & Brochon, J. C. Picosecond tryptophan fluorescence of thioredoxin: evidence for discrete species in slow exchange. *Biochemistry* **28**, 3383–3398 (1989).
10. Riedl, J. et al. Lifeact: a versatile marker to visualize F-actin. *Nat. Methods* **5**, 605–607 (2008).
11. Zimmermann, T. Spectral imaging and linear unmixing in light microscopy. in *Microscopy Techniques* (ed. Rietdorf, J.) 245–265 (Springer, 2005); https://doi.org/10.1007/b102216

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