Chapter 4
Fluorescence Lifetime Imaging Microscopy

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Fluorescence lifetime imaging microscopy (FLIM) is an imaging technique based on the analysis of exponential decay rates of fluorophores. In FLIM, the contrast relays on the lifetime of individual fluorophores rather than their emission spectra and/or intensity [1]. The first reports of FLIM date back to the late 1990s [2]. In FLIM, a very short pulsed laser is used to excite a sample. For fluorescence detection, a laser-scanning confocal microscope uses a pinhole effect, blocking all light from outside the focus. The emitted photons from the fluorophores pass back through an objective lens, and are then spectrally separated using a dichroic beam splitter. After the beam splitter, photons are quantified by highly sensitive detectors (photodiode or photomultiplier). FLIM is based on the differences in the excited state decay rates from fluorescent samples.

Generally, light absorbing molecules emit photons when returning from an excited state (S1 or subsequent states) to their ground state (S0). The transition from the excited state to the ground state is characterized by several parameters, such as, (a) the fluorescence spectrum, (b) the ratio of the total number of emitted photons to the number of absorbed photons, and, (c) the fluorescence lifetime. The lifetimes of most naturally occurring fluorophores typically range within the order of several nanoseconds (10^{-9} s). In order to measure these lifetimes, it is essential to use excitation pulses which are considerably shorter than the decay time of the fluorescence. Most FLIM systems are equipped with pulsed lasers which have a picosecond (10^{-12} s) or femtosecond (10^{-15} s) pulse duration. According to the "Stokes shift", the emitted fluorescence has a longer wavelength and less energy compared to the excitation light. Gabriel Stokes postulated in 1852 that there is a difference in energy between the maxima of the absorption and emission spectra. The fluorescence

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lifetime is a very useful parameter in imaging fluorescence, as it represents the change in fluorescence over time and is highly sensitive to the environment.

The measured mean fluorescence lifetimes, τ_m, are mapped spatially within the microscope image. The acquired nanosecond excited state lifetime is independent of the fluorophores concentration, sample thickness, or excitation intensity. As such, the signal is more robust than intensity-based methods. Fluorescence lifetimes depend on environmental parameters such as pH, ion concentration, and molecular binding or proximity to other energy acceptors. Therefore, FLIM allows for functional imaging. Super-resolution techniques of fluorescence imaging microscopy even allow for spatial resolution of single molecules [3].

Most conventional FLIM microscope systems operate in the visible wavelength range. Some systems operate in the near infrared (NIR) range (400–900 nm) and the latest developments use deep ultraviolet (UV, 240 nm) [4] or shortwave infrared (IR, 1700 nm) wavelengths [5].

There are several techniques to record the incoming photons. In Time-Correlated Single Photon Counting (TCSPC), FLIM intensity values are recorded in subsequent time channels [6, 7]. These time channels contain the number of recorded photons after the excitation pulse, which allows to acquire the decay curve for an individual point or pixel in the image. Fourier-domain FLIM does not record individual photon detection events, but rather records the fluorescence signal as a waveform [8].

Currently, there are several approaches for image analysis in TCSPC FLIM. The simplest way to analyze the data is by using a single exponential decay model. In this model the data is represented by a single decay time. In most cases, however, the decay profiles are more complex and have to be modelled by two or three exponential functions. These models contain several decay components and amplitude coefficients. The FLIM image is then presented as a color-coded image where decay times, or decay components, are represented by different colors.

FLIM can also be used to differentiate fractions of the same fluorophore in diverse states of interaction with its environment [9]. Several properties of FLIM can be exploited to gain additional molecular information or information on the fluorophores environment. Förster resonance energy transfer [10], or FRET, is based on energy transfer from the first molecule, or the donor molecule, to the second molecule, or the acceptor. FRET results in a quenching of the donor fluorescence and therefore decreases the decay time of the donor molecule. Because the energy transfer rate decreases with distance on a molecular scale, this technique has been very useful in molecular cell biology as FRET allows verifying whether molecules are physically linked on a nanometer scale [11, 12]. The use of FRET is currently the most frequent FLIM application.

Other applications include fluorescence quenching by oxygen, which is a quencher for a large number of fluorophores [12] or fluorophore quenching by ions such as Ca^{2+} and Cl^{-}, both of which are found in vast amounts in the neuronal system.

In autofluorescence FLIM, autofluorescence serves as an intrinsic, label free contrast. Because a mixture of intrinsic fluorophores can usually be found in a tissue

sample, the decay profiles of tissue autofluorescence are multiexponential with lifetime components ranging from below 100 ps to several nanoseconds. In this case, multiple lifetimes may be observed as there are more than one fluorophore present in the sample or there are different chemical interactions with one fluorophore. Autofluorescence FLIM shares many similarities with FLIO. However, in the future, other FLIM applications such as identification of fluorescent dyes or nanoparticles may be combined with FLIO imaging and may then find clinical application.

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