Quantitative and sensitive live-cell imaging with FIDSAM (fluorescence intensity decay shape analysis microscopy) for biomaterials research

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Cell adhesion on biomaterial surface is crucial for the regeneration and function of clinically viable cells and tissues. In turn, the cellular phenotypes following the mechanoochemical transduction of adherent cells on biomaterials are directly correlated to the biophysical responses of cells. These responses are expected to be mediated by changes within the cytoskeletal architecture and are often visualised by transduction of cells with gene constructs encoding for fusion proteins for which a protein of interest is tagged with a fluorescent protein (FP). Fluorescence microscopy has become an invaluable tool for cell biology, mainly due to advances in electronics, optics and fluorescent labels. Although outstanding sensitivity down to the observation of single molecules can be reached by these fluorescence-based techniques, for biological tissue interference of autofluorescence background with the label signal is a major problem. This autofluorescence may cause artefacts and lead to image misinterpretations. Accordingly, strong effort is made to eliminate autofluorescence, e.g. by spectral or temporal discrimination. However, in many cases both, the fluorescence spectrum as well as the fluorescence lifetime of the autofluorescence signal, quantitatively overlaps with that of the target fluorescence. We developed a novel method called FIDSAM (fluorescence intensity decay shape analysis microscopy), which utilizes the characteristic shape of fluorescence intensity decay of typical label dyes such as fluorescent fusion proteins to discriminate target signal from autofluorescence background. The technique benefits from the fact that the method is robust and insensitive to local changes of the fluorescence emission, such as changes in the fluorescence lifetime due to the individual chemical environment of the fluorescence label. Thus, FIDSAM is a robust tool for fluorescence image improvement and offers the ability to quantitatively avoid image artefacts. Combined with fluorescence lifetime imaging (FLIM), FIDSAM allows for studying the biomechanical adaptation of cells on biomaterial surfaces. In this chapter, we describe recent developments of confocal fluorescence microscopy with FIDSAM-FLIM for live studies of FP expression systems.

Keywords cell architecture; cell imaging, life cell imaging, FLIM, FIDSAM

Diverse networks of tissues and cells that have largely different physical properties build up the human body. This characteristic implies that a biomaterial, which performs well in one site, may not be convenient in another part of the body. Implants for therapeutic applications are most commonly made of metals, ceramics or polymers. For the development of new biomaterials for use as implants or for use in tissue engineering cellular processes such as adhesion, ingrowth, survival and fate of cells are relevant parameters to be investigated. Physical and chemical properties of the material not only influence cells’ ability to adhere and spread but also provide guidance cues for cell differentiation or tissue formation [1]. Furthermore, surface structure and topography at the nano- and microscales have been shown to influence function and fate of adherent cells [2]. Cell adhesion and cell spreading to a substrate are fundamental for cell survival and proliferation. Within tissues, first cell-substrate contacts are made by cellular heterodimeric transmembrane integrin receptors whose extracellular domains bind to peptide ligands of extracellular matrix (ECM) proteins such as Arg-Gly-Asp (RGD) in fibronectin. Binding of the adapter protein talin to β integrin intracellular subdomains leads to talin activation followed by accumulation of a large number of proteins including vinculin, paxillin, tensin and many others, which are all involved in the dynamic association with actin filaments [3]. In adherent cells, at least three types of integrin–based protein complexes are formed that are different in their molecular composition and size. Focal complexes are small (0.5-1 µm) dot-like contacts localized at the edges of lamellipodia while focal adhesions (FA) are elongated (2-5 µm in length) structures that associate with the actin- and myosin-containing bundles (stress fibres) at the cell periphery [4]. FAs can further mature into fibrillar adhesions (1-10 µm) in the central region of the cell [5]. Intracellular proteins and soluble factors such as growth factors regulate traction forces that are generated by actomyosin interaction and actin polymerisation. Through stress fibres via FAs cell traction forces are transmitted to the ECM where they direct many cellular functions including ECM reorganisation, cell proliferation, migration, differentiation and cell apoptosis [6]. For instance, distinct changes in cell architecture together with changes in quantity of FAs upon osteogenic differentiation have been described recently [7]. FAs not only function as adhesion sites but also contribute to adhesion-dependent signal transduction. Cells are able to sense the mechanical properties of their surrounding environment and subsequently regulate several cellular processes [8]. In line with that, it has been shown previously that the application of a local force by pipette manipulation stimulates centripetal growth of FAs [9]. Such reinforcement of FAs involves the recruitment of new adhesion proteins and complex elongation [9].

Since cell adhesion is fundamental for cellular survival and function it is a critical parameter for initial assessment of newly developed biomaterials [10]. Cell-substrate adhesion can be quantified by mechanical techniques or non-mechanical methods. Using atomic force microscope (AFM) cantilevers is only one of many mechanical methods to
measure the detachment force of an adherent cell on a material [10, 11]. With regard to non-mechanical methods cell adhesion can be monitored by various microscopy techniques. Fluorescence labelling of representative adhesion or cytoskeleton molecules (e.g. vinculin, paxillin and actin) together with light microscopy is nowadays widely used to investigate adhesion capacity and cytoskeletal architecture of cells on biomaterials. By applying specific antibodies immunohistochemistry enables the detection of specific antigens within cells (see Fig. 1). When a detectable marker (e.g. fluorescein or rhodamine red) has been conjugated to the antibody of interest this antibody can be monitored within the cell. However, for immunohistochemistry cell samples are generally fixed, which restricts the obtained results to snapshot information on the studied proteins.

Fig. 1 Immunofluorescent labelling of vinculin in SaOs2 cells cultured for 4 days on ceramic surfaces [12]. (Scale bar: 20 µm)

Genetically encoded FPs help overcome such limitation. The discovery, gene cloning and heterologous expression of green fluorescent protein (GFP) from the jellyfish Aequorea victoria revolutionized cell biology. Expression of a FP results in visible fluorescence without any additional cofactors. Direct fusion of FPs to proteins of interest by molecular cloning techniques enables high molecular specificity and renders investigation of protein function in living cells possible. A large number of gene constructs has been created in the past two decades. For identification of FA complexes many protein therein can be labelled. The adhesion protein vinculin is often used for monitoring FAs as it first exists in almost every cell type and second is present in both types of FAs, e.g. central and peripheral. Nevertheless, several other proteins are used to study their function for cell adhesion. For instance, GFP-labelled α-actinin and paxillin have been previously used to study the organisation of stress fibres and FAs [13] while formation and reorganisation of actin bundles during spreading of REF52 cells was revealed by time-lapse microscopy of GFP-actin [14]. Nowadays, a broad range of FPs with different excitation and emission spectra is available. Due to that diversity, distinct labelled proteins within single cells can be monitored online (as shown in Fig. 2).
Stably or transiently transfected cells have gained attention in biomaterial research to study cell adhesion capacity. Titanium or titanium alloys are commonly used for bone fixation as well as joint implants in clinics and several groups attempt to optimise cell adhesion to titanium surfaces. The influence of structural alterations of the titanium surface (by polishing, machining or blasting) to cell adhesion has been studied recently [15]. The distribution of different integrins in human primary osteoblast and MG-63 cells was visualised by immunohistochemistry. Fibrillar adhesions occurred on polished and machined titanium. Further, GFP-tagged vinculin was used to examine the number, size and dynamic behaviour of FAs in living MG-63 cells that were cultured on titanium surfaces with different degrees of roughness [16]. In another study, titanium was coated with a thin plasma polymer layer to assist initial osteoblastic cell adhesion [17]. Within this work the formation of actin cytoskeleton as well as paxillin organisation was monitored in MG-63 cells by immunohistochemistry. In addition, vinculin distribution was analysed in GFP-vinculin transfected MG-63 cells showing that FAs are strongly organised on functionalized titanium but sparsely developed on pure titanium (Fig. 3).
Fig. 3 Vinculin organization in GFP-vinculin-transfected MG-63 osteoblasts after cultivation for 60 min on functionalized Ti surfaces. Note that the vinculin containing FAs are strongly organized in cells on plasma polymerised allylamine (PPAAm) and polyethylenglycol-diacid-collagen (PEG DA-COL), which is comparable to the collagen (COL) control. In contrast, on pure Ti vinculin is sparsely developed after such short time of cultivation. Inserts are magnified views of indicated areas (arrows) [17].

In another example for life-monitoring of FAs the relative role of surface chemistry and surface topography on human osteoblasts has been studied by dos Santos et al. in 2007 [12]. Two distinct types of nano roughness of phosphate ceramics were produced and some samples were gold-sputter coated to mask surface chemical effects. Immunofluorescent labelling of actin and vinculin revealed no differences in cell spreading of cells cultivated on different nano-topographies. However, strong topographical effects on cell proliferation and differentiation were verified. Within the last years, the creation of surfaces with defined densities of ligand binding peptides (e.g. RGD) has become possible [18, 19]. It has been shown in several studies that the cell’s ability to adhere and spread is strongly affected by the kind and also the quantity of adhesive ligands. In the majority of studies, fluorescently labelled FA proteins are used to evaluate the cell’s ability to adhere and these results suggest the use of fluorescently labelled adhesion proteins to represent a well suitable approach to probe the capability of materials to promote cell adhesion. In addition to the reasons discussed above, this is emphasised by the sensitivity of this method, which nowadays even allows the detection and spectroscopy of single molecules [20-23]. Moreover, due to the high spatial resolution of around 250 nm in (confocal) microscopy measurements, adhesion processes can be visualised on a subcellular level.

However, monitoring can be hindered by strong non-specific fluorescence signals from the tested material itself, which have to be excluded for a precise fluorescence based analysis of the adhesion process. In biological samples, these non-specific signals are referred to as autofluorescence background, which contributes as a dominant signal over the entire spectral range. Accordingly, strategies have to be developed to distinguish autofluorescence signal from the target signal of the distinct visible FPs fused to the proteins of interest [24].

As discussed above, the autofluorescence quantitatively overlaps with the FP marker-signal; hence straightforward spectral filtering using e.g. bandpass filters is not effective (Fig. 4a). Accordingly, different parameters that can help to discriminate autofluorescence contributions had to be defined. This introduces the field of novel types of microscopy applications where spectral information is recorded along with image acquisition. These approaches can be referred to as spectro-microscopy. In conventional fluorescence microscopy methods, mainly the spatial resolved intensity information recorded from a fluorescent sample is used for image acquisition. In spectro-microscopy, further parameter
spaces are added to the evaluation to increase the information content extracted from a sample. One prominent approach uses the time evolution of fluorescence intensity. This so-called fluorescence lifetime imaging microscopy (FLIM) records the fluorescence lifetime for every image pixel and arranges the product of intensity and fluorescence lifetime in a false-colour FLIM image [25]. Although the information content is drastically increased and different fluorescent species can be distinguished by their fluorescence lifetime, FLIM often fails in cell-biological samples harbouring high autofluorescence contribution. This is mainly due to the fact that the autofluorescence lifetime is often found to be in the same range as for typical FPs (Fig. 4b). Additionally, the fluorescence lifetime, although being a characteristic parameter of the investigated fluorescent species, is strongly affected by the local physico-chemical environment. Whereas this environment can be assumed to be a steady and controllable value in vitro, the picture entirely changes for live cell conditions [26, 27]. Here, the local environment is highly anisotropic due to the complex composition of a living cell, consisting of a multitude of different compartments. As a result, it is impossible to assign a distinct fluorescence lifetime value to a certain fluorescence marker but rather a range of values must be considered, frustrating a robust discrimination of autofluorescence and label signal. Hence, it is desirable to use different characteristics of autofluorescence for discrimination.

![Fig. 4](image)

**Fig. 4** a) Spectral overlap of autofluorescence occurring in fibroblasts with typical FP label dyes. b) Overlap and variance of fluorescence lifetimes of plant autofluorescence (red bars) and eGFP (enhanced green fluorescent protein, green bars) as a typical example for cellular label dyes.

One characteristic becomes accessible when considering the individual fluorescence statistics of fluorescence and autofluorescence. Fluorescence emission statistics can be investigated by studying the fluorescence evolution after excitation with a short laser pulse. This technique is referred to as time correlated single photon counting (TCSPC) and is commonly applied in conventional FLIM measurements. The principle of TCSPC shall be shortly introduced in the following.

A TCSPC experiment starts with an excitation pulse (e.g. generated by picosecond diode lasers) shorter than the fluorescence lifetime of the investigated molecules. Thus, the fluorophores are excited and a measuring clock is started. TCSPC is a digital technique, counting photons emitted from the sample for different time intervals relative to the excitation pulse. This is done using a fast detector (e.g. a photomultiplier or an avalanche photo diode (APD)) connected to a time-to-amplitude-converter (TAC) electronics, which can be regarded as a fast stopwatch. During the experiment the sample is repetitively excited by a pulsed light source and every excitation pulse is optically or electronically monitored to produce a start signal, which is used to trigger a voltage ramp of the TAC. This ramp, which is realized by loading a capacitor, is stopped as soon as the first photon from the sample is detected and an output pulse is generated whose voltage is proportional to the time elapsed between start-pulse and stop-signal. Subsequently, a multichannel analyzer (MCA) converts the voltage to a time channel using an analogue-to-digital (AD)-converter and the single photon arrival times are stored. Summing up over many pulses the MCA builds up a histogram of counts versus time channels [28]. Although the basic principles underlying the TCSPC-technique are straightforward, there are a number of specific requests, which are indispensable for a TCSPC-measurement. One requirement for reliable experimental data is that not more than 1 to 2 photons are generated per 100 excitation pulses, or, in other words, only 1-2% of the excitation pulses should result in a counting event. This limitation is due to the present electronics, which only allow for the detection of the first arriving photon per excitation pulse. As a consequence, once a photon is detected, the dead time of the electronics prevents the detection of another photon. Recalling that fluorescence emission is a random process, more photons are emitted at early times than at late times. Hence, if many photons arrive on the detector and only the first one is counted, the intensity decay is distorted to shorter times due to overrepresentation of photons detected at the beginning of the measurement. This behaviour is known as the so-called pile-up effect.
The histogram of photon arrival statistics obtained from the TCSPC measurement can now be addressed analytically. For that, a convenient approach is to assume the fluorescence time statistics as a decay reaction $S_1 \rightarrow S_0$ with a kinetic constant $k = \frac{1}{\tau}$ and $\tau$ as the fluorescence lifetime. One can describe the rate equation according to

$$-\frac{d[S_1]}{dt} = k[S_1]$$

and integration yields

$$[S_1] = [S_1]_0 \cdot \exp(-kt)$$

Taking $[S_1]$ proportional to the measured intensity and replacing $k$ by $\frac{1}{\tau}$, equation 2 reads

$$I = I_0 \cdot \exp\left(-\frac{t}{\tau}\right)$$

As can be directly deduced from equation 3, the intensity evolution of the fluorescence ideally exhibits a purely monoexponential characteristics, given that exactly one species of emitters, e.g. one defined population $[S_1]$ with only one definite kinetic constant $k$ contributes to the measurement. The picture entirely changes if a multitude of $i$ different emitters is considered. Here, formally the reaction reads $\sum_i S_{1,i} \rightarrow \sum_i S_{0,i}$ and equation 3 becomes multiexponential according to

$$I_{\text{mean}} = \sum_i I_{0,i} \cdot \exp\left(-\frac{t}{\tau_i}\right)$$

Equation 4 is a summation over a multitude of individual decay statistics and thus reflects the measured TCSPC histogram of autofluorescent cell areas.

Obviously, these considerations imply that autofluorescence and target signal from e.g. FP can be discriminated solely by their photon statistics independent from their spectral shape or their fluorescence lifetime.

This correlation between photon statistics and the specificity of the contributing emitters is utilized in a novel kind of fluorescence microscopy, named “fluorescence intensity decay shape analysis microscopy” (FIDSAM) [29]. Due to the differences in the photon statistics of autofluorescence and target emission, the shape of the intensity decay curves of the different contribution also differs. Hence, the intensity decay measured by TCSPC can be described more accurately by a monoexponential behaviour (equation 3) the more target signal contributes to the decay curve. On the contrary, the TCSPC curve will show stronger deviations from this monoexponential behaviour when a large fraction of the signal originates from autofluorescence. This is because autofluorescence is comprised of the signal of a large number of different emitters, each one following different decay statistics.

Accordingly, for a quantitative discrimination of the autofluorescence, it is merely required to determine the magnitude of deviation of the measured signal from a monoexponential behaviour (which reflects the exclusive contribution of target chromophores). Such quantification is feasible by fitting an analytical expression according to equation 3 to the experimental data normalized to intensity. Practically, the expression in equation 3 is fitted to a measured decay curve and the fitting is optimized using a least-square-fit routine. Now, the quality of the fit is a direct measure for the autofluorescence contribution and can be quantified by the error square $\chi^2$ according to

$$\chi^2 = \sum_{i=1}^{n} \left[\frac{N(t_i) - N_c(t_i)}{N(t_i)}\right]^2$$

In equation 5, $\chi^2$ reflects the sum of the differences between all measured values $N(t_i)$ and the fitted values $N_c(t_i)$. For a best quality fit, $\chi^2$ approaches 1 and rises for increasing deviations. As a result, discrimination or suppression of autofluorescence can be obtained by multiplying the original fluorescence intensity value by the inverse error value $\frac{1}{\chi^2}$. With this, image pixels originating from samples or sample areas with high autofluorescence contribution are divided by a high number, resulting in a low numerical value. Contrarily, areas with dominant target signal contribution are divided by a small value, approaching 1 for an ideal fit, and thus remain largely unaffected by the correction procedure. For data acquisition, a standard confocal fluorescence microscope with FLIM-extension can be used and a monoexponential decay function according to equation 3 is fitted to every data point. The obtained error values $\chi^2$ are
then arranged in a corresponding spatially resolved error-image, which in turn is inverted and multiplied with the original intensity image. As a result, areas with high autofluorescence contribution are divided by a high value and are thus attenuated relative to areas with small or no autofluorescence interference.

As a major advantage, the FIDSAM technique benefits from the adjustable strength of image correction. As the procedure is multiplicative, the introduction of a variable pre-factor allows for a flexible adaptation of FIDSAM to the recorded images. This possibility is not provided by any established method for autofluorescence discrimination so far. For example, spectral unmixing, a technique which utilizes differential variations in the spectral shape of the recorded fluorescence signal, is not capable of a precise adjustment of the correction strength and is thus limited in its applicability and complex sample systems where autofluorescence is dominant and/or exhibits a strong spectral overlap with the target signal can hardly be addressed by this method [30].

Further, it is worth to mention that FIDSAM is not only limited to marker dye systems exhibiting a perfect monoexponential decay but can also be applied to label dyes such as CFP (cyan fluorescent protein), which intrinsically decays biexponentially. Although the CFP signal generates error values $\chi^2 > 1$ and thus FIDSAM reduces the target signal in the corrected image, autofluorescence still exhibits a stronger deviation and thus is suppressed to a significantly greater extent. As a result, even for biexponentially decaying dyes such as CFP, FIDSAM is feasible to significantly enhance the obtainable image contrast by selectively discriminating autofluorescence contribution.

To study the FA sites of human fibroblasts discussed in the beginning it is suggestive to apply FIDSAM to CFP labelled vinculin.

Fig. 5a shows an original confocal intensity image of a human fibroblast, expressing CFP-fused vinculin under control of a CMV-promotor. Accumulation of fluorescence emission is found in distinct areas near the cell periphery as well as in more central regions of the cell.

The picture significantly changes when FIDSAM is applied to the image depicted in Fig. 5a. After the generation of the error image (Fig. 5b), FIDSAM was applied 4-fold (Fig. 5c) and 7-fold (Fig. 5d), respectively. Depending on the FIDSAM strength, clearly some, albeit not all, intracellular located features disappear. This finding is interesting and crucial for quantitative data handling as well as for subsequent investigations. Typically, one would expect the majority of FAs sites to be located at outer cell areas, where they indeed are found in high concentrations. However, FIDSAM reveals that on the one hand most of the intracellular fluorescence signal disappears and must hence be attributed to origin from autofluorescence. On the other hand, distinct features remain unaffected and are still visible in the corrected FIDSAM image (Fig. 5c and d). Obviously, these features are due to CFP-vinculin emission, which is located in the central region of the cell.
As the fluorescence decays have been recorded for FIDSAM determination it is obvious to combine FIDSAM with FLIM, e.g. using the FIDSAM corrected intensity image and colour-code including the fluorescence lifetime information.

![False colour confocal FLIM image of a human fibroblast expressing vinculin as a CFP-fusion protein.](image)

**Fig. 6** a) False colour confocal FLIM image of a human fibroblast expressing vinculin as a CFP-fusion protein. b) FLIM-FIDSAM image of a) after 7-fold correction. The spherical features with shorter lifetime in a) are largely suppressed by application of FIDSAM and are therefore attributed to autofluorescence.

Fig. 6 depicts the original FLIM image (Fig. 6a) and the 7-fold corrected FLIM-FIDSAM image (Fig. 6b). In the original image, the small features showing up inside the cellular boundary clearly show a different fluorescence lifetime than the emission at the cell membrane. In the FLIM-FIDSAM image, the vesicles with shorter fluorescence lifetime are selectively suppressed, demonstrating that they do not originate from the CFP-vinculin but rather correspond to unspecific background-signal due to autofluorescence. This finding is also of great importance for studies based on fluorescence resonance energy transfer (FRET) where the excitation energy of a donor chromophore, e.g. CFP, is transferred non-radiatively to an adjacent acceptor chromophore, e.g. YFP (yellow fluorescent protein). Due to the strong distance dependence of the FRET-process, obeying an inverse 6th power law, this technique is highly sensitive and distances on a scale of typically 5-15 nm can be measured and quantitatively determined [28, 33]. This way, it is possible to investigate force-dependent molecular interaction between FA proteins using confocal FRET-studies. Here binding events can be visualised by a changed FRET efficiency and be quantified e.g. by a reduced donor fluorescence lifetime $\tau_{DA}$. The importance of applying FIDSAM is emphasized when considering that a shortened fluorescence lifetime of CFP in a FRET pair may be in the same region as the autofluorescence lifetime found for fibroblasts (Fig. 6). Therefore, fibroblast autofluorescence might be misinterpreted as a signal originating from CFP undergoing FRET. This is due to the fact that in FLIM-based FRET investigations the reduced donor lifetime $\tau_{DA}$ in presence of an acceptor is used as an evidence for an energy transfer to an acceptor. Hence, from the original FLIM image one would judge efficient FRET- interaction in the small intracellular features due to the shortened fluorescence lifetime. However, FIDSAM reveals that these features do not correspond to specific CFP-donor signal but are due to autofluorescence. Accordingly, in the FLIM-FIDSAM image these features vanish and do no longer corrupt FRET analysis and thus data interpretation.

We presented a novel microscopic approach, combining confocal microscopy with time resolved FIDSAM-FLIM techniques as a valuable tool for quantitative live cell imaging. Moreover, we demonstrated future applicability of this technique for highly sensitive functional studies of cellular adhesion sites.

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