Unsupervised Fluorescence Lifetime Imaging Microscopy for High-Content and High-Throughput Screening

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Running title: Unsupervised FLIM for HTS and HCS
Abbreviations:

CCD, Charge-Coupled Device
CV, Coefficient of Variation
EGFP, Enhanced Green Fluorescent Protein
EYFP, Enhanced Yellow Fluorescent Protein
FACS, Fluorescence Activated Cell Sorting
FLIM, Fluorescence Lifetime Imaging Microscopy
FRET, Förster Resonance Energy Transfer
HCS, High Content Screening
HTS, High Throughput Screening
ICAS, Image Cytometry for Analysis and Sorting
ICCD, Intensified Charge-Coupled Device
Summary

Proteomics and Cellomics clearly benefit from the molecular insights in cellular biochemical events that can be obtained by advanced quantitative microscopy techniques like fluorescence lifetime imaging microscopy and Förster resonance energy transfer imaging. The spectroscopic information detected at the molecular level can be combined with cellular morphological estimators, the analysis of cellular localization, and the identification of molecular or cellular sub-populations. This allows the creation of powerful assays to gain a detailed understanding of the molecular mechanisms underlying spatio-temporal cellular responses to chemical and physical stimuli. This work demonstrates that the high content offered by these techniques can be combined with the high-throughput levels offered by automation of a fluorescence lifetime imaging microscope setup, capable of unsupervised operation and image analysis. Systems and software dedicated to Image Cytometry for Analysis and Sorting represent important emerging tools for the field of proteomics, interactomics and cellomics. These techniques could soon become readily available both to academia and the drug screening community by the application of new all-solid-state technologies that may result in cost-effective turnkey systems. Here, the application of this screening technique to the investigation of intracellular ubiquitination levels of α-synuclein and its familial mutations that are causative for Parkinson’s disease is shown. The finding of statistically lower ubiquitination of the mutant α-synuclein forms supports a role for this modification in the mechanism of pathological protein aggregation.
Introduction

Above and beyond the isolation and identification of proteins, the field of proteomics faces the challenges of detecting protein cellular localization and quantifying molecular states such as protein conformations, protein-protein interactions and post-translational modifications. In the past decade, Förster Resonance Energy Transfer (FRET) and Fluorescence Lifetime Imaging Microscopy (FLIM) have proven to be instrumental for the quantitative imaging of these biochemical states in single cells (1). Similarly, the analysis of different cellular populations (cellomes) will also benefit from these imaging methods. Quantitative multi-parametric microscopy is a very young field in which advances in liquid/sample handling robotics and information technology are gradually being integrated into automated microscopes (2,3). These automated imaging systems merge the high content image information with the high-throughput volumes provided by their automation and unsupervised operation.

Screening techniques have now reached (ultra)High-Throughput levels, i.e. they are capable of performing more than $10^5$ assays per day in microliter volumes. Such a high throughput is necessary for applications where (bio)chemical libraries are tested, e.g. for drug discovery and interactomics research (4). Although the advance in throughput scale is necessary, it is often accompanied by low information content. Evidently, multi-parametric detection at high numbers would present a powerful tool. Moreover, the screening reproducibility and estimators need to respect comparatively high quality standards, e.g. coefficient of variations (CV) and z-scores should not exceed 5% and should be higher than 0.5, respectively.

High-Content (HCS) applications typically involve the quantitative and multi-parametric analysis of the effect of analytes or other perturbing conditions on cellular behaviour (5). The understanding of molecular mechanisms underlying disease, for instance, requires high-
resolution information as the screens target the cellular and/or sub-cellular level. Such applications aim at the monitoring of molecular pathways; the localization and interactions of biomolecules and their altered behaviour in response to drugs or pathogens.

An automated fluorescence lifetime imaging microscope capable of unsupervised operation was developed to provide the basis for a scalable screening platform that combines high-throughput levels and high-content information gained from quantitative multi-parametric imaging.

Fluorescent protein engineering offers a wide variety of genetically-expressible fluorescent biosensors, *e.g.* for the detection of ion concentration, pH, molecular oxygen, proteolytic and chaperone activity, and ubiquitination, many of which can be quantitatively detected by fluorescence lifetime sensing (6). Their exquisite selectivity is derived from the fact that these biosensors can be targeted to specific proteins-of-interest, organelles and other sub-compartments of the cell. In addition, a wide variety of site-specific orthogonal protein labeling strategies using synthetic dyes is available nowadays, *e.g.* FlAsH, ReAsH, SnapTag, Halo (promega), CoA binding (6). The availability of commercial systems for automated fluorescence imaging is constantly growing (7-9). Moreover, recent works demonstrate the usefulness of time-resolved fluorescence assays in screening (10,11).

In this work, an automated FLIM that is based on state-of-the-art technology, *i.e.* intensified charge-coupled-devices (ICCD), is described. We recently introduced new all-solid-state technologies (12,13) that will enable the construction of cost-effective and turnkey systems that do not require specialized knowledge for their maintenance and operation.

In light of the presented results and novel technologies, we envisage comparatively cheap and simple high-throughput and high-content quantitative screening platforms to become available in
the near future. These systems would provide a substantial impulse to the recent and actively expanding fields of drug discovery, interactomics, cellomics and proteomics.

**Experimental procedures**

**Microscopy**

The automated microscope used in this work is based on a frequency-domain FLIM setup that is described elsewhere (14) in more detail (see also supplemental figure 1 and 2). The core of the system consists of a motorized Axiovert200M (Carl Zeiss Jena GmbH, Jena, Germany) and an ICCD (PicoStar, by Lavision GmbH, Göttingen, Germany). Additionally, a high-resolution CCD camera (imager Compact, by Lavision GmbH) and a SwissRanger-2 Time-of-Flight imager (CSEM SA, Zürich, Switzerland) can be mounted on the binocular phototube output port. The samples were scanned by translating the computer-assisted microscope stage (LSTEP by Märzhäuser GmbH & Co. KG, Wetzlar-Steindorf, Germany). Focus, optical port selection, shutters, objective revolver, filter turret and filter wheel are also motorized. The microscope is equipped with HbO (ATTO-Arc 100, by Zeiss) and XbO (HAL 100, by Zeiss) lamps, an Argon Ion laser Innova 300C Argon laser (Coherent Inc., Santa Clara CA, USA), a solid-state laser Compass 405nm (by Coherent Inc.) and a light-emitting-diode illumination module NSPB500S (Nichia Corp., Japan). The excitation source can be freely chosen and switched. Specific exciter and emitter filter cubes are used to select different fluorophores in a sample. In the present work, Rhodamine 6G, EGFP and EYFP were excited by the 488 nm laser line of the Argon ion laser. In the case of the screening of REACh-ubiquitination of GFP-α-synuclein, GFP was excited with the 458 nm line of the Argon ion laser. The filter turret hosts a beam splitter, a low efficiency reflector, and two filter cubes whose emitter, dichroic and exciter were: i) band-pass 440-460
nm, long-pass 470 and band-pass 480-500 nm; ii) band-pass 490-510 nm, long-pass 515 nm and band-pass 520-550 nm; iii) band-pass 460-480 nm, long-pass 493 nm and band-pass 505-530 nm (AHF Analysentechnik AG, Tübingen, Germany). These filter cubes were used for the experiments show in figures 6, figure 2 and figure 1, 3, 4, and 5, respectively. All above-mentioned features were integrated in a virtual microscope environment that allows the automation of the entire imaging process. This environment was controlled by in-house developed software programmed in the DaVis suite (LaVision GmbH). Schematics are available on http://www.quantitative-microscopy.org/pub/uFLIM.html and in the supplemental material.

Screening protocol

Initially, the user defines the type of screening and calibrates the microscope with a fluorescence lifetime standard positioned at the sample plane. At regular intervals, the microscope compares the calibration parameters with the phase and demodulation of the light source by a low efficiency reflector positioned in the filter turret to correct for possible drifts in the relative phase of the system over time. The dynamic calibration offered by this internal reference does not require the sample to be removed or the interaction of the user. The user can define an arbitrary number of virtual acquisition channels. The microscope is not equipped with a single detector with spectral and lifetime capabilities, but acquisition channels are defined by i) the light source (laser, HbO, XbO, or LED module), ii) the filter set (the turret hosts 4 different filter cubes and a filter wheel in front of the HbO lamp that is fitted with 8 excitation filters), iii) and the detectors (ICCD for FLIM or a high-resolution CCD), and are selected via software. With a profile chosen, the microscope selects and presents a series of field-of-views on which the user may manually focus. These focus landmarks are used in the auto-focussing routines by interpolation over the
sample. Subsequently, the system scans the sample and computes the exposure time of the
detector in order to avoid its saturation. For this, two images are acquired at opposite phases (0°
and 180°) with a low exposure time (typically 20ms) and a pixel-by-pixel average intensity and
fluorescence lifetime is computed using the rapid lifetime determination algorithm (15). Based
on these parameters, the platform decides whether to image the current field-of-view, \textit{i.e.}
whether a fluorescent object is present, and computes the optimal exposure time. If requested,
the microscope can refine the focus position by the use of an iterative “staircase” procedure (16)
with a focus-score based on sampling at half the Nyquist frequency as described in (17). As this
process is rather time-expensive, it is ideally limited to a small number of fields-of-view, \textit{e.g.}
those containing objects identified by certain search criteria. This system could be equipped with
autofocus hardware (18) for improved acquisition throughput. The microscope then switches to
the next virtual channel to acquire the images and stores the data in the memory. The images of
each field-of-view are stored on mass storage devices during the movement of the stage between
fields. In the case of time-lapse screening, this procedure is repeated after a user-defined time lag
in order to follow a process on a large number of cells over time. The recorded object time- and
spatial- coordinates allows time-dependent measurements to be made for each individual object.

\textit{Data-Analysis}

A second computer analyzes the images that are acquired by the platform. The data is transferred
in a local-area-network, and is processed on-line. The results are typically available on-screen
within a few seconds after the acquisition. A stand-alone tool for remote monitoring of the
screening activities was developed in Microsoft Visual Basic, allowing the fully unsupervised
acquisition/analysis process to be monitored by local-area-network access or an internet connection (see supplemental material).

This process returns the ensemble of original images, the processed intensity and lifetime maps, a low-resolution global map of the sample and an array of estimators for every imaged object. Every object is flagged with its relative position in space and time. The features of single objects that are analyzed comprise: intensity (in different spectral ranges), homogeneity of the intensity (coefficient of variation), fluorescence lifetime and the LiMA heterogeneity estimator (14), and morphological estimators, e.g. area, perimeter, elongation and roundness. Other analyses that can be performed on the data include invariant moment analysis, and the analysis of intensity/lifetime in specific cellular compartments that are identified by morphological estimators or fluorescent labelling. User-assisted statistical software provides access to the unsupervised read-out. These routines allow object counting of the imaged sample, and the extraction of sub-populations from the ensemble by inspection of data clusters in combinatorial bi-dimensional histograms. Both the unsupervised batch processing and the supervised statistical analysis software were developed in Matlab (Mathwork, Natick MA, USA). Part of the Matlab code and further information can be downloaded from www.quantitative-microscopy.org/pub/uFLIM.html.

Sample Preparation

EGFP was purified from a liquid culture of transformed BL21DE3 E. coli bacteria by immobilized metal chromatography using the 6xHis tag of the protein. Rhodamine 6G was prepared at a concentration of 200 µM in distilled water from a 10 mM methanol stock solution. A 1536 multi-well plate (SensoPlate by Greiner Bio-One GmbH, Frickenhausen, Germany) was
loaded using an automated liquid handling station (Freedom EVO by TECAN). Potassium iodine was added at the indicated concentrations (see Fig.1) to the wells prior to plate loading.

For the imaging of bacterial colonies, BL21DE3 E.coli bacteria were transformed with the pRSET(B)::YFP vector and plated on an agar layer that was cast in a custom-built plate with removable teflon walls to facilitate their removal before imaging. This allows the entire cultured surface to be exposed to the objective when the plate is mounted on the stage.

CHO cells were transfected with pEYFP and/or pEGFP vectors using the Effectene 2000 lipid formulation according to the protocol provided by the supplier (QIAGEN GmbH, Hilden, Germany). Liposomes containing either pEGFP vector, pEYFP vector or pEGFP and pEYFP vectors were prepared by incubating the respective DNA or a 1:1 mixture of both DNAs with the Effectene reagent. The four wells of a LabTek chamber slide with glass bottom were transfected with the two individual DNA-lipid solutions, the mixed DNA-lipid solution and a mixture of both individual DNA-lipid solutions. Rat striatal CSM14.1 cells were transfected with pcDNA3.1 vectors encoding the genes for wild type α-synuclein, the A30P, or A53T mutant in a four-well Labtek chamber slide. These constructs were co-transfected with the recently described REACCh2-ubiquitin fusion construct (19). The cells were allowed to express the fluorescent proteins for 48 h, upon which the cells were fixed in 4% (w/v) formaldehyde in PBS, washed and mounted in mowiol.

Results

Unsupervised FLIM for High-Throughput

The ultra-High Throughput standard (uHTS), requiring more than $10^5$ assays per day in microliter volume, defines the highest current throughput level of screening platforms. The 1536
multi-well plate is a format that allows these assays to be performed under the given criteria when read in ~20 minutes.

Figure 1 shows the intensity and lifetime maps of a 1536 multi-well plate whose wells were loaded with different fluorescent solutions. Pairs of rows, i.e. sets of 96 wells, were loaded with purified EGFP, Rhodamine6G (R6G) and potassium iodine (KI) by liquid handling robotics. The EGFP and R6G solution exhibited fluorescence lifetimes of ~2.6 ns and ~4.2 ns, respectively. Under these experimental conditions, EGFP is brighter than R6G (Fig.1A, first and last rows, respectively). Additionally, quenching of R6G with decreasing concentrations of potassium iodine (63 mM, 50 mM, 38 mM, 25 mM, 13 mM and 0 mM) was used to generate a gradient of intensities and lifetimes. The comparison between panel A and B shows that lifetime detection provides excellent contrast and that the readout is independent of the probe concentration. In fact, differences in brightness due to pipetting errors or illumination inhomogeneity are not present in the lifetime map. Fig.1C shows the averages and the standard errors of the 8 different groups of wells that contained the same solutions. The R6G quenching curve is resolved with high accuracy. The coefficients of variation were in the range of only 1-2% and the standard errors on the mean are in the picosecond range.

The parallel imaging of 4 wells per field-of-view with a low magnification objective (5x) allowed the complete multi-well plate to be imaged in ~20’ (~0.7s per well). Therefore, these instruments could, in principle, perform at uHTS levels when equipped with sample handling robotics and rapid auto-focusing hardware.

**Scalability**
One of the advantages offered by an automated microscopy platform is its straightforward scalability. Light sources, filters, detectors and objectives can be selected to match the sample requirements. Figure 2 shows images of a custom-built 14 x 9 cm bacterial plate. *E.coli* bacteria transformed with pRSETB-EYFP were plated at the maximal achievable density that is compatible with imaging: ~20,000 colonies per plate. The agar plate can be screened in ~90’ by imaging ~1900 field-of-views (corresponding to a total of ~7600 exposures) using a 5x objective. The segmentation of single objects (Fig. 2, panels B, D and E) allows the retrieval of fluorescence intensity (Fig.2A), lifetime (Fig.2C) and morphological estimators for each bacterial colony.

The images shown in Figure 1 and 2 were acquired using the rapid lifetime determination algorithm. This algorithm allows higher throughputs than the common multi-point phase acquisition used in frequency-domain FLIM as it requires the acquisition of only two opposite-phase images. Lifetime heterogeneity and photo-stability can be obtained upon acquisition of more phase-dependent images, but at the cost of (~ half the) acquisition speed (20).

**High-Content Screening**

Figure 3 shows images of a 4-well chamber microscope slide on which CHO cells were cultured that express EGFP and EYFP. The first two wells were transfected with liposome preparations containing only pEGFP or pEYFP, respectively. The second two wells were co-transfected by mixing pEGFP and pEYFP vectors in the same liposomes (EGFP+EYFP) or by mixing the two separately prepared homogeneous liposome solutions containing pEGFP and pEYFP (EGFP/EYFP). 15,000 cells were imaged in 90’ using a 20x objective. The cells were segmented and analyzed for intensity and lifetimes by the unsupervised software. Here, 6 phase-images
were acquired for the computation of the lifetime heterogeneity by Lifetime Moments Analysis (LiMA)(14).

Figure 3A shows the lifetime distributions (circles) based on single cell statistics of the cells identified in the four different wells together with their Gaussian fits (solid lines). Unlike the other samples, the lifetime distribution of the “EGFP/EYFP” sample does not seem to be monovariate (back). Although the average lifetime of the “EGFP+EYFP” and “EGFP/EYFP” mixtures are similar, i.e. 2.26 ± 0.10 ns (N = 5372) and 2.29 ± 0.15 ns (N = 3705), respectively (average ± standard deviation), only the former distribution can be fitted by a single Gaussian.

Therefore, the “EGFP”, “EYFP” and “EGFP+EYFP” samples represent homogeneous cell populations that exhibit single Gaussian distributions (solid lines). The “EGFP/EYFP” can be fitted by three Gaussian distribution components (black solid line) whose averages and standard deviations were constrained to those retrieved from the homogeneous conditions (see supplementary material for further details). Therefore, it follows that only about 30% of the cells received the two different liposomes that were present in the preparation.

Figure 3C-D summarizes the average lifetime and relative brightness in each well in a representative region of 5 x 25 fields-of-view. Because of differences in protein expression levels, the brightness (Fig.3B,D) does not provide a robust estimator for the comparison of the samples. On the other hand, the fluorescence lifetimes (Fig. 3A,C) clearly distinguish between the different transfection conditions. The cells expressing EGFP or EYFP alone exhibit a fluorescence lifetime of 2.13 ± 0.10 ns (mean ± standard deviation, n=2488) and 2.43 ± 0.11 (n=3472). The relative brightness of the two samples shows a bi-modal distribution showing that cells express more EGFP than EYFP under the conditions used.
The lifetime heterogeneity estimator also shows differences between the four samples: 81 ± 12 %, 92 ± 12 %, 86 ± 11 % and 85 ± 12 % for “EGFP”, “EYFP”, “EGFP+EYFP” and “EGFP/EYFP”, respectively. Bi-dimensional histograms of the fluorescence lifetime heterogeneity versus the average fluorescence lifetime of each cell (Figure 3E) show the correlation between these two estimators. In fact, EYFP has a higher lifetime and a higher heterogeneity than EGFP. Such analysis can be extended to other pairs of estimators for the analysis of subpopulations in a manner comparable to FACS analysis (see supplemental material).

Figure 4 shows the individual cells in this field-of-view that are marked with a circle in fig.3C. Each cell was identified by an image processing routine that consists of automatic background subtraction and automatic threshold detection, followed by a watershed algorithm and a morphological mask operation. Segmented objects with fluorescent intensities below 5% of the CCD dynamic range were masked out and ignored. Object classification was performed off-line by supervised software. Fig. 4A shows the result of this process; segmented cells are color-coded and rejected objects are presented in a nonlinear grey-level map in order to highlight their low fluorescence levels. Fig. 4B-C shows the lifetime map of the successfully segmented cells. Although the cells that were transfected with EGFP and EYFP alone exhibit average lifetimes that differ only by 300ps, up to 95% of cells can be correctly classified as either EGFP or EYFP by a linear separation of the two populations. The two co-transfections “EGFP+EYFP” and “EGFP/EYFP” exhibit average lifetimes and a distribution broadness that differ by only 30 ps and 50 ps, respectively. However, the high number of analyzed cells permits the retrieval of the weight of the three underlying distributions.
Finally, all four populations exhibited identical eccentricity; 63 ± 15 %, 62 ± 16 %, 62 ± 16 %, 62 ± 16%. This transfection-independent quality proves that differences in brightness do not bias the other estimators.

*A high-throughput high-content cellular assay for ubiquitination by FRET*

Ubiquitination of α-synuclein in the rat striatal CSM14.1 cell line was investigated using a FRET-based assay (19). A total of 871 cells were imaged with ~1140 fields-of-view using a 40x objective. GFP fusion proteins of α-synuclein, and its familial mutations A30P and A53T, were co-expressed with ubiquitin fused to a non-fluorescent YFP mutant (REACCh2). The transfer of energy from the GFP donor to the REACCh acceptor is highly efficient due to their optimized spectral overlap. Ubiquitination is quantified by the occurrence of FRET between the GFP-tagged α-synuclein protein and the REACCh2-labeled ubiquitin that causes a reduction in the GFP lifetime. Four samples (control, wild-type, A30P and A53T mutants) were imaged in 45 minutes each, including the focusing procedure. The GFP lifetime in the absence of FRET was determined in a control sample that expresses GFP-α-synuclein alone and amounted to 2255 ± 9 ps (average ± SEM, N = 191). The stability of the measurement was verified by re-screening of this sample at the end of the measurement in a smaller number of cells. The retrieved lifetime was statistically identical to the control (2248 ± 8 ps, N = 35). The lifetime of wild type GFP-α-synuclein in the presence of ubiquitin-REACCh2 was reduced to 1817 ± 13 ps (N = 219) and significantly lower than for the two α-synuclein mutants whose lifetimes were not statistically different from each other (A30P: 1928 ± 13 ps, N = 270; A53T: 1914 ± 14 ps, N = 156). FRET efficiencies were computed by the reduction in lifetime relative to the control sample. The distribution of FRET efficiencies computed on a cell-by-cell basis for all α-synuclein constructs...
is shown in Fig 6A. An increase in FRET efficiencies is observed when ubiquitin-REACCH is co-expressed with the respective GFP-labeled α-synuclein construct. Higher FRET efficiencies are accompanied by the broadening of their distribution due to the presence of multiple interaction modes and/or ubiquitination levels. Additionally, the average distributions of FRET efficiencies are broader than the distributions of the single cells (Supplemental Figure 7), indicating the presence of separate ubiquitination levels also between individual cells. Gaussian fitting of the composite FRET efficiency (ubiquitination) distribution shows the presence of 3 populations for wild type α-synuclein (a fraction of 0.33 at 12% FRET, 0.39 at 21%, and 0.28 at 27%). The mutant α-synuclein forms loose the highest FRET efficiency population and the remaining populations are redistributed in favor of the lowest population (A30P: 0.6 at 12% and 0.4 at 16%, A53T: 0.56 at 11% and 0.47 at 19%). In contrast to the GFP-YFP co-mixing experiment shown in Fig 3-5, a reversed correlation of the coefficient of variation (14) with the lifetime exists. This is caused by the increase of heterogeneity with increasing FRET, and presents an additional quality criterion for FRET.

**Discussion**

FRET operates at intermolecular distances on the scale of protein dimensions (<10nm) and exhibits sensitivity to changes in the Ångstrom range. FLIM provides a non-invasive, fast and quantitative FRET measurement, thus giving access to molecular information like protein-protein interactions and conformational changes. Furthermore, lifetime sensing was used for the quantification of oxygen content, ion concentration and pH and can be used to map biochemical events in living cells (6), proving its value for molecular proteomics studies. The diversity of available synthetic dyes with sensing capabilities for different small molecules and conditions...
can be exploited by FLIM to create new sensitive and reproducible assays for a variety of cellular functions. This holds particularly true for those dyes that respond with otherwise difficult to calibrate quantum yield changes, and that are now avoided in favour of ratiometric dyes.

Such detailed and quantitative information is equally important for the life sciences and the screening industry. It was shown (see fig.1) that an automated FLIM, capable of unsupervised operation, provides very high throughput, with good reproducibility (CV<5%) and sensitivity (high z-score). An assay is considered robust when its statistical z-score exceeds 0.5 (21). With the coefficient of variation in our studies, this stringent statistical requirement can be fulfilled with 20% lifetime difference detected in a single well. High sensitivity and reliability is of crucial importance for the FRET-based detection of protein-protein interactions and protein conformational changes. Furthermore, assays can be performed in a variable environment, e.g. in cells, and in “homogeneous” assay formats that do not require washing steps, by the virtue of the independence of the fluorescence lifetime from fluorophore concentration. FLIM screening platforms could be used for the validation of protein-protein interaction found by other (u)HTS approaches. One such application example is shown for the screening of ubiquitination levels of α-synuclein and its familial mutations that are causative for Parkinson’s disease. With its high-throughput, automated FLIM systems could be directly used for the screening of fluorescently labelled genomic banks or drug libraries.

Our experiments also exemplify that the scalability of an automated microscope allows the analysis of samples that do not respect a standardized format: we showed the unsupervised imaging of microtiter plates (fig.1), bacterial plates (fig.2) and microscope slides (fig.3-6). Other
samples like tissue slices, electrophoresis gels, DNA or protein arrays, and nanotiter plates could also be easily accommodated.

Figure 2 shows the screening of bacterial colonies. Besides screening for optimization of fluorescent proteins and fluorescent biosensors by random mutagenesis, fluorescence lifetime based assays could be performed in bacteria as a biological model system that carry the advantage of the simplicity of sample handling, biochemistry, and retrieval of genetic/proteomic compositions.

The microscope stores the relative position of each imaged object. The sample can therefore be revisited iteratively for real-time data analysis. In addition to the “inventory” use of the platform in cell screening, the platform can therefore also be used to “hunt” for rare events with the aim of sample retrieval. Single colonies, cells or cellular sub-populations could be isolated, for instance, by photogelation procedures (22) or laser microdissection and pressure catapulting (23) techniques. The protein or genetic content of the objects with specific lifetime properties can then be analyzed by the relevant techniques.

These two modes of operation are generally known as Image Cytometry for Analysis and Sorting (ICAS) (22). ICAS is suitable for adherent cells and tissues, where flow cytometric techniques cannot be used. Our work shows that the highly informative and sensitive fluorescence lifetime parameter can be used for the selection of cells for ICAS.

Figure 3 demonstrates the unsupervised cellular imaging and data analysis of extended surfaces. Data acquisition with 6 phase images was performed here to analyse the lifetime heterogeneity (14) and to compensate for photobleaching (24). In the case of FRET imaging, the quantification of lifetime heterogeneity by Lifetime Moment Analysis can provide a measure of the molecular
fraction that undergoes FRET, *e.g.* the relative concentration of interacting proteins and their average intermolecular distance. When photobleaching and lifetime heterogeneity of the fluorophores can be neglected, the rapid lifetime determination algorithm can be used that requires only two phase-dependent images. Under these conditions, the screening of an entire 4-well Labtek chamber would take a third of the current time, *i.e.* 30’. The maximal cell density and transfection efficiency that allow single cells to be distinguished amounts to ~100,000 cells in this format. Therefore, a maximum of 200,000 cells/hour can be screened with a 20x objective. The screening can be repeated over time, by imaging extended surfaces or a user-defined group of cells (data not shown). This enables the measurement of temporal responses over a high number of cells.

Figure 3, 4 and 5 exemplify how cellular sub-populations can be analysed by imaging single cells. The differences between the two co-transfection conditions used would be impossible to resolve when only the averages over these large numbers of cells were considered. The analysis of cell populations is important for the understanding of the regulation and molecular mechanisms of biological events as biological models are usually heterogeneous. The capability of screening and segmenting diverse cellular populations, combined with the possibility to detect protein-protein interactions can offer a significant advantage for the fields of cellular proteomics and interactomics.

Quantitative multi-parametric microscopy and automated unsupervised microscopy are comparatively young techniques that attract a growing number of industrial and academic research groups. This work represents an advance in the combination of these technologies and demonstrates that current technologies can be used for the construction of an unsupervised FLIM.
system for high-throughput and high-content screening. Several commercial automated systems could be adapted for lifetime sensing, immediately offering a powerful tool for the screening community. The experiments presented in this work represent well-defined benchmarks for the characterization of the quality of the data that is generated, and for the application of software solutions for the detailed statistical analyses that can be performed. FRET assays enjoy an increasing popularity in the life sciences, and present the major application of our platform. The feasibility of sensitive FRET assays on our platform is demonstrated by its high quality and sensitivity. Figure 5 shows that a lifetime difference of 300ps can be clearly separated. Furthermore, taking into account the CV, 95% of the cells could be successfully classified. This difference corresponds to a FRET efficiency of approximately 12% with fully separated distributions. In the same experiment, three populations differing by only ∼6% were still reliably separated. The ubiquitination assay shows that also differences of ∼4% can be distinguished (wild-type versus A30P mutant) with high statistical significance (p < 0.01, t-student) by the use of the same fluorescence assays used in conventional microscopy.

This remarkable resolution in the biochemical event of protein ubiquitination is only achieved by the automation of the lifetime microscope, combining high throughput with high content information; large cell numbers in the sample were subjected to the uniquely quantitative determination of FRET by lifetime microscopy. The cell-based statistics identify differences in the ubiquitination of disease-related mutant forms of the α-synuclein protein. These forms are ubiquitinated less than the wild type α-synuclein. All three α-synuclein proteins seem to share two basal states of ubiquitination, but the wild type protein possesses an additional high ubiquitination state that drives the average significantly upwards. The cellular response to the presence of α-synuclein ubiquitination substrates is thus intrinsically heterogeneous, a fact that
would be lost if this modification is measured by biochemical means, with imaging approaches that do not resolve the individual responses (e.g. plate readers), or by manual acquisition of only a few cells. As ubiquitination of unwanted proteins, leading up to their proteasomal digestion, is an integral part of the detoxification machinery of the cell, the observed altered behaviour of the familial disease-causing α-synuclein mutants is important for the understanding of the pathophysiology of Parkinson’s disease. This novel findings are in agreement with the slower proteolytical degradation of A53T α-synuclein observed in pulse-chase biochemical experiments (25). Furthermore, the clinical hallmark of this disease is the presence of intracellular inclusion bodies, called Lewy bodies, which contain ubiquitinated α-synuclein (26) and ubiquitin-proteasomal dysfunction is generally considered to be an important feature of Parkinson’s disease (27). However, it is not known in what way these observations are causally connected. The high sensitivity afforded by our screen is crucial as, given the progression of Parkinson’s disease over decades, even minor impediments of α-synuclein degradation could favour the formation of aggregates. The mutations have been suggested to inhibit proteasomal activity (27), but differences in their ubiquitination levels were never reported by conventional biochemical methods. For the first time, it was shown that ubiquitination level of α-synuclein can be quantitatively imaged in cells and that the mutants are significantly less ubiquitinated. As the mutants exhibit an increased tendency toward self-aggregation, giving rise to neurotoxicity, their decreased ubiquitination might indicate that their altered proteolytic processing contributes to aggregation. On the other hand, ubiquitination might represent a mechanism that prevents α-synuclein from aggregation. These issues warrant further research.
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Figure 1. High-throughput screening and reproducibility. 8 groups of 96 wells in a 1536 multi-well plate were loaded with 1) enhanced green fluorescence protein (EGFP), 2) a mixture of rhodamine 6G (R6G) and EGFP, and 3-8) a gradient of R6G quenched with potassium iodine at 63 mM, 50 mM, 38 mM, 25 mM, 13 mM and 0 mM concentration, respectively. Two empty wells served as a control for plate autofluorescence. The plate was screened in less than 20’ (5x objective) with high sensitivity and reproducibility. Note that the standard errors and coefficients of variation of the fluorescence lifetimes (table and panel C) are in the range of picoseconds and 1-2%, respectively.
Figure 2. High throughput and system scalability. The relative brightness (A) and fluorescence lifetimes (C) of a large agar surface (9 x 14 cm), plated with ~20,000 E.coli bacterial colonies expressing EYFP, was imaged in ~1 hour with ~1900 fields of view (5x objective). A higher magnification (B) shows the single colonies. Each field of view (D, E, squares in B) is segmented (D), and the fluorescence lifetime of single colonies is assigned (E). The spatial coordinates of each segmented colony is stored to allow subsequent higher resolution imaging or sample retrieval.
Figure 3. High content screening. A four-well Labtek chamber was seeded with CHO cells, transfected with EGFP and/or EYFP, and imaged in ~90' with ~600 fields of view (20x objective). The wells were transfected with a vector encoding EGFP (EGFP), EYFP (EYFP), cotransfected with liposomes containing a mixture of EGFP and EYFP vectors (EGFP+EYFP), and co-transfected with a mixture of EGFP liposomes and EYFP liposomes that were produced
separately (EGFP/EYFP). Panel A shows the distributions of lifetimes computed over the single cells (~ 15,000 in total - circles) and the data fitted with Gaussian functions (solid lines); the EGFP/EYFP sample is best represented by a sum of the other three populations. Panel B shows the distribution of brightness. Panel C and D show the synthetic representation of the average lifetime and brightness, respectively, in each field of view of a 5 x 25 field of view region of interest. The circle in the EGFP well indicates the field of view that is shown in more detail in figure 4. Panel E show the bi-dimensional distribution of lifetime heterogeneity plotted versus the average lifetime of the four samples. The black arrows indicate the averages of the EGFP and the EYFP samples.
Figure 4. Unsupervised cellular image processing. Single cells are identified by segmentation in each field of view (A). Cells are identified by intensity and size; objects that are classified as possible cells are color-coded, all other objects are shown in a nonlinear gray-level map. The fluorescence lifetimes (B) are computed only on the segmented cells. Different morphological and spectroscopic estimators can be computed for each object. Panel C shows the distributions of lifetimes in each cell (blue curve) compared with the lifetime distribution computed on the segmented pixel ensemble (red curve). The average lifetime of the pixel ensemble is 2.13±0.22 ns.
Figure 5. Analysis of α-synuclein ubiquitination by high-content FRET screening. A four-well Labtek chamber containing CSM14.1 cells transfected with GFP-α-synuclein alone (black) or co-expressing REACH2-ubiquitin together with GFP-α-synuclein (grey) or GFP-fusions of the A30P (orange) or A53T (green) mutant of α-synuclein was imaged in ~3 hours with ~1140 fields of view (40x objective). The distribution of lifetimes computed over the single cells (A, 871 in total) and the average lifetimes retrieved from the different samples (B, ±SEM) are shown using the same color-coding. The middle row shows two-dimensional histograms of the coefficient of
variation versus the lifetime for each cell in the samples (C). Warmer colors indicate higher numbers of cells. The lower row shows the presence of sub-populations in the FRET efficiency distributions shown in A. These populations were extracted by Gaussian fitting without constraining the parameters and using a more than two-fold improvement of the $\chi^2$ value as criterion to include additional Gaussian components. The original distribution is shown in black (with original data points as red open circles), the fitted Gaussians are included as grey curves.
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