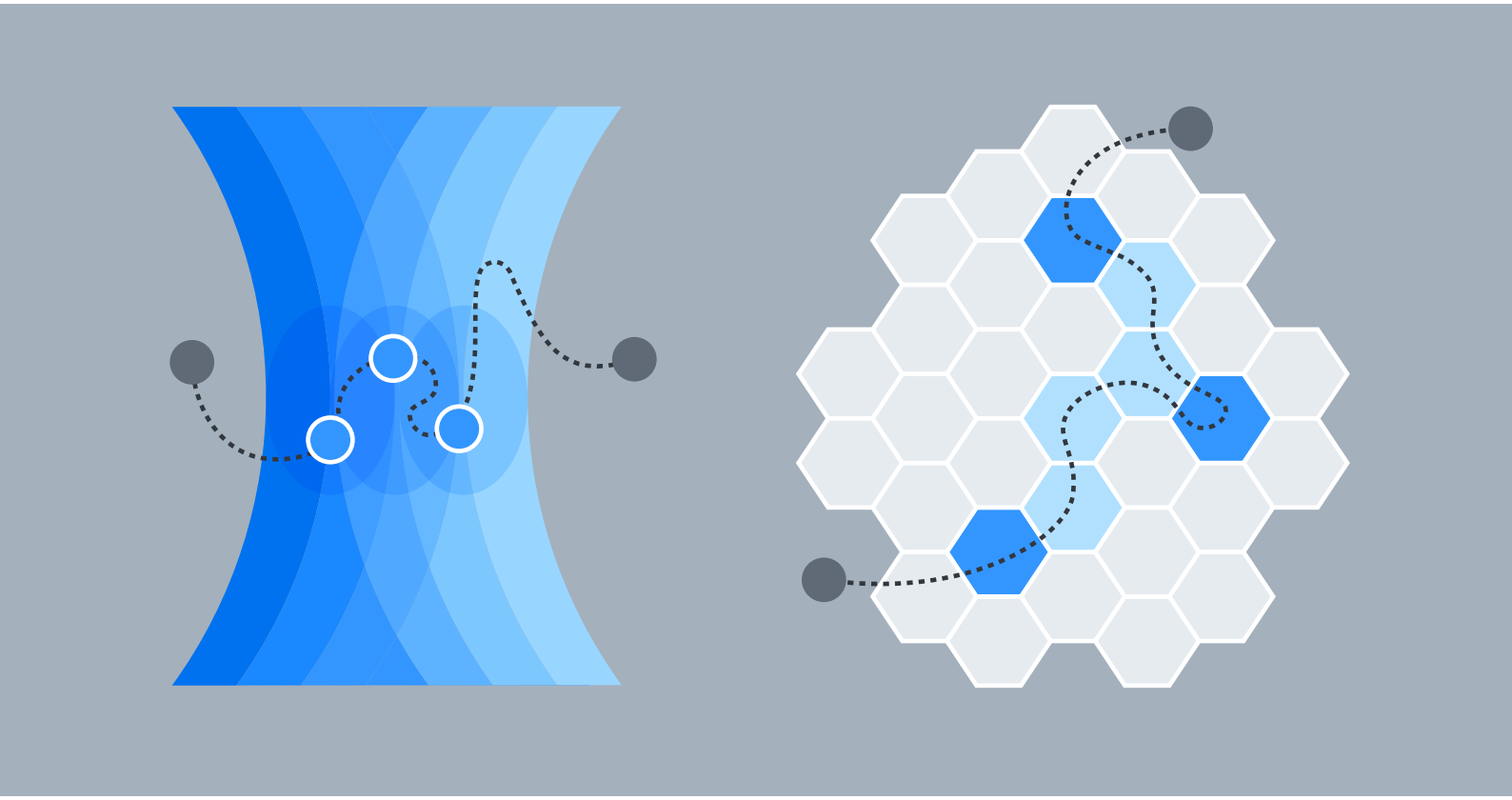
Follow dynamic biological processes and reveal spatial molecular characteristics

ZEISS Dynamics Profiler



ZEISS

Seeing beyond

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Introduction

The scientific drive to understand life in ever greater detail continues to push forward the development of microscopy technology. For this reason, technologies providing super-resolution microscopy have been explored in the past decade, resolving smaller and smaller structures, and observing previously unknown details even in living samples. Today's turn-key fluorescent microscopy systems allow scientists to investigate subcellular, sub-organelle and molecular structures in more detail and complexity than ever before. As expected, these different super-resolution approaches come with their own boundaries and pitfalls, such as high light exposure of the samples which can influence the behavior of living samples by phototoxic effects. Investigation of fast living processes is limited by the relatively slow image acquisition that occurs when resolution is pushed to its limits. Technologies that allow capture to the single molecule level must commonly restrict visualization to only a few members of the molecule population of interest, while the behavior of the majority remains invisible. While microscopy imaging experiments remain an important contributor to the investigation of biological molecular processes withing living organisms – many findings are based on non-microscopic in-vitro work, using techniques such as biochemistry, x-ray modelling, computer-modelling, or binding-essays. These methods are rather detached from the *in vivo* situation of the protein in question. While image-based microscopy cannot always provide all the answers when it comes to molecular behavior, microscopy-based technology and techniques do allow deeper insights into molecular characteristics *in vivo* within their environment.

To explore the dynamics of molecular behavior, particle tracking and Fluorescence Recovery After Photobleaching (FRAP) experiments are popular approaches. For particle tracking, individual single molecules must be resolved. As a result, it is better suited to sparse molecules with moderate to slow movement or only a subset of the whole molecule population is represented. Faster processes can be measured with FRAP experiments. Here a defined region of interest (ROI) is bleached to remove fluorescent signal from the labelled protein. The fluorescent signal within the ROI is recovered by fluorescently labelled protein from the adjacent areas which moves into the bleached region. Average values of a large population of molecules can be acquired using this method. However, the aggressive bleaching procedure can produce negative side effects in the living sample, sometimes even influencing the measured behavior of the protein of interest. Very often, each single FRAP experiment requires a new cell or even organism, so generating comparable data within a single cell or organism are not always achievable. Similar approaches are based on photoactivatable or photoconvertible fluorescent proteins; instead of eliminating fluorescent signal from a ROI, the fluorescent signal is generated in a region within the sample and the spreading of the signal is recorded. This approach not only allows the recording of dynamics but also the overall direction of the movement. While less aggressive than FRAP, the procedure generally allows only one such experiment per cell or organism and delivers only coarse-to-non-spatial specificity of the dynamic behavior.

More information and detail about molecular characteristics within a sample can be collected by using Fluorescence Correlation Spectroscopy (FCS) experiments (see Info Box 1 "FCS Principles" and [4]). Measuring only single excitation spots within the sample, allows the determination of molecular behavior at high spatial accuracy. Since this approach is a lot more sensitive, it allows several measurements within a single sample, even within a single cell. Using this approach, the protein of interest can be characterized by its diffusion mobility and concentrations in different compartments of the sample (e.g., cytoplasm versus nucleoplasm). A single FCS experiment can reveal several aspects of molecular characteristics and answer questions such as:

- How many molecules are there? What is the concentration of the protein of interest in this part of the sample?
- How great is the mobility of the molecules?
- What is the composition of the molecules – do they remain monomers, form dimers, multimers or aggregates?

Despite the potential power of this approach for generating diverse and detailed information about molecular characteristics, conventional FCS measurements are not commonly used by every biologist. This is mainly due to the restriction that conventional FCS can only be conducted if the concentration of the fluorescently labelled molecules is very low and expression levels are normally optimized for imaging experiments, which requires much higher brightness than conventional FCS allows. Measuring intensity fluctuations is also quite far away from a standard microscopy experiment since there is no actual image capture but rather the generation of numbers, graphs, and statistics. This is why FCS can seem disconnected from the imaging experiments that are normally done with a microscope.

Molecular characteristics within imaging experiments of living cells, 3D cell culture (e.g., organoids or organ transplants), or organisms are therefore often omitted even though the information about the molecular concentration could be a valuable addition to the live cell imaging dataset.

This technology note introduces the Dynamics Profiler – a unique option to collect information about molecular concentration and dynamic characteristics for a wide variety of samples and experimental setups for which such measurements were not possible before (Figure 1). Furthermore, it will show how the Dynamics Profiler is able to provide you with unique and new information about your sample. Dynamics Profiler will enhance and expand experimental opportunities for experiments conducted on living cells or organisms, microfluidic setups including organs on a chip, giant unilamellar vesicles (GUVs) or similar model membranes, cellular condensates generated by liquid-liquid phase separation or any passive or active movement of molecular labels in a fluid environment.

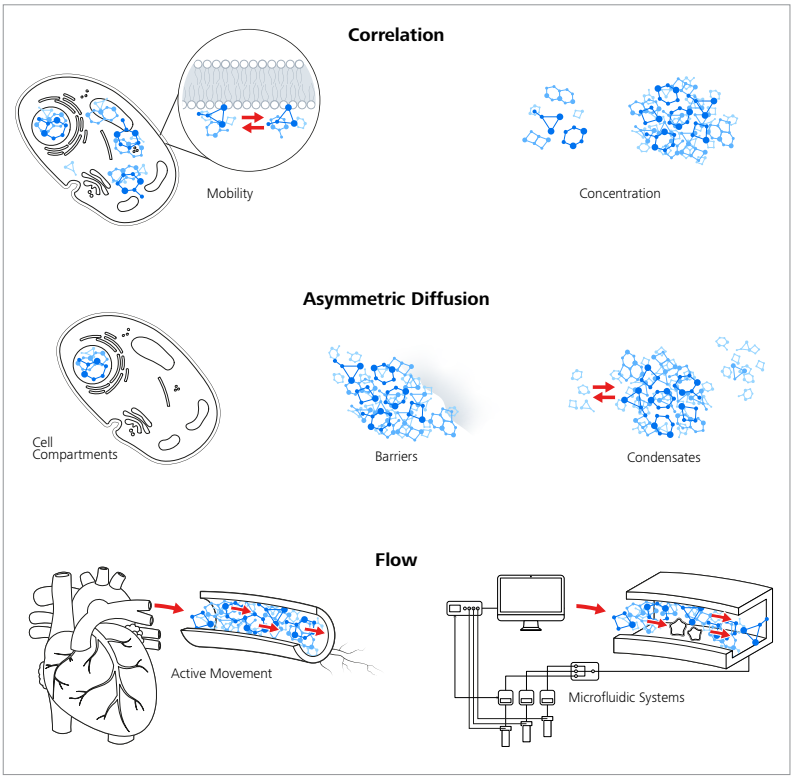


Figure 1 *An overview of Dynamics Profiler applications*

Fluorescence Correlation Spectroscopy (FCS) Principle (Box 1)

Fluorescence Correlation Spectroscopy (FCS) is a technique to measure the dynamics of moving fluorescently labelled particles. It is commonly used with point scanning systems such as multiphoton or confocal Laser Scanning Microscopes. A stationary excitation volume, created by an objective lens focused laser beam, is positioned in a living or otherwise dynamic sample and fluorescent intensity fluctuations are recorded over several seconds, resulting in an intensity trace measurement (Figure 2). These intensity fluctuations are generated by single fluorescently labelled particles that pass through the excitation volume due to diffusion or other means of movement. The acquired intensity trace I(t) is now compared with itself by shifting in time τ, which is called a temporal autocorrelation.

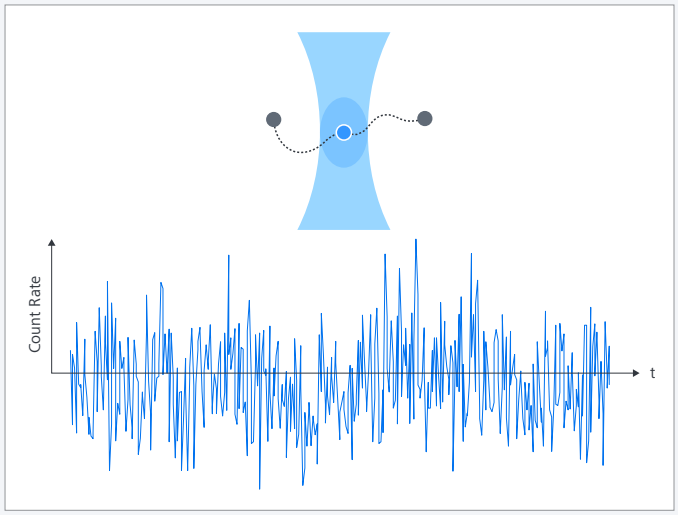


Figure 2 *A stationary excitation volume, created by an objective lens focused laser beam (top), is positioned in a sample and fluorescence intensity fluctuations are recorded for this spot over several seconds, resulting in an intensity trace measurement (bottom).*

$$G(\tau) = \frac{< I(t) \cdot I(t+\tau) >}{(< I(t) >)^2}$$

The resulting correlation graph (Figure 3) holds information on the number of molecules and diffusion time.

Diffusion time: The intensity trace is shifted by lag time τ against itself. As long as it remains similar to itself, the value of G(τ) remains high. When the similarities start to decrease along τ, the graph will descend towards 1 at the G(τ) – axis. This is why fast movement results in a steep correlation curve, while slower movement results in a slow descending correlation curve.

Number of molecules: The fluorescently labelled particles move into and out of the excitation volume, at any time an amount of these particles will be present in the volume. If the overall concentration of the particles is low, only a few will be in the excitation volume and a single molecule moving in and out will

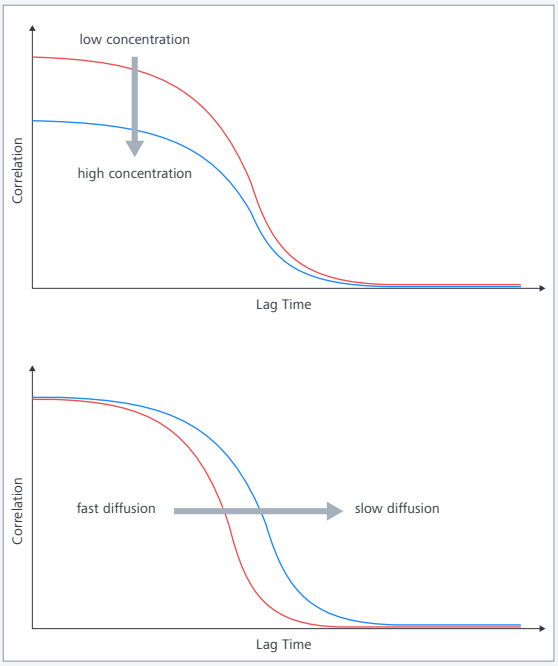


Figure 3 *The correlation graph holds the information on molecule numbers (amplitude) and diffusion time (convergence time to the baseline).*

result in a relatively high change in intensity levels. If the concentration is high, a single molecule added or removed from the excitation volume will only lead to a relatively small change in intensity. This is why the correlation graph starts at a higher value of G(τ) at low concentrations and at a lower value at high concentrations respectively (Figure 3).

To determine the diffusion coefficient and the molecular concentration, the dimensions of the Point spread function (PSF) of the specific measurement is needed in addition to the diffusion time and the number or molecules respectively. To achieve this a so-called ωr calibration (see chapter "Calibrations") is performed and the results are integrated into the fitting process.

The correlation graph needs to be fitted to a mathematical model which describes the dynamics behavior of the molecules, e.g., two different components freely diffusing in a volume. The better the potential characteristics of the particle of interest are known the better this model can be adapted. In general, it is a good rule that data interpretation gets easier with increased knowledge about the potential behavior of the sample. Dynamics Profiler provides a variety of fit models that address many common molecular dynamics behaviors (for details see Appendix A).

Dynamics Profiler – advantages for live imaging experiments

Dynamics Profiler is using fluorescence correlation spectroscopy (FCS) methods in combination with the Airyscan area detector to provide new information about dynamic processes *in vivo*, *in vitro* or in material samples. At the same time, it tackles many of the challenges that prevented such experiments with conventional FCS until now. The whole measurement process and analysis is combined in a coherent workflow of a wizard that ensures best data quality and easy recognition of new information (see "Workflow Dynamics Profiler"). The combination of bright sample measurement, easy to follow workflows and additional spatial information enables FCS novices and experts to collect novel information about molecular concentration and dynamics to add to their investigations of living samples. Different from current FCS setups, Dynamics Profiler acquires molecular traces with all 32 detector elements of the Airyscan detector (Box 2). Every measurement collects data fit for all three types of included analysis, and for individual correlation and analysis schemes. In this way, sample exploration and the potential of generating new findings is available without needing additional light exposure or measuring time.

Dynamics Profiler Principle (Box 2)

Dynamics Profiler uses the FCS principle in combination with the Airyscan detector [1]. Airyscan comprises circularly arranged 32 individual channels forming an area detector, used for sensitive super-resolution and parallel detection (Airyscan Multiplex mode) imaging.

The FCS principle remains unchanged (see Box 1) but with the difference that each individual detector element records an intensity trace, adding unique spatial information to the FCS measurement. While data from all 32 detector elements is recorded, Dynamics Profiler analysis uses the information from the innermost 19 elements only (< 1.0 AU). These detector elements provide both sufficient spatial distance and the best signal-to-noise ratio. The outer-most elements collect less signal and have therefore a low signal-to-noise ratio.

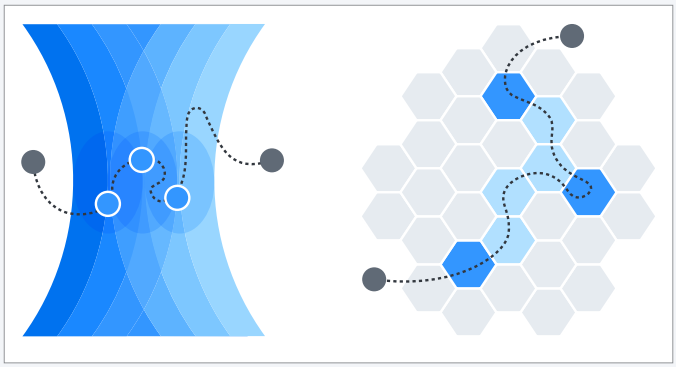


Figure 4 *Dynamics Profiler principle*

Dynamics Profiler includes three integrated analyzing options, two of which utilize the additional layer of spatial information. The different analyzing options are addressed here by referring to the tab names in the ZEN software (Figure 5). All used analysis formulas can be found in Appendix A.

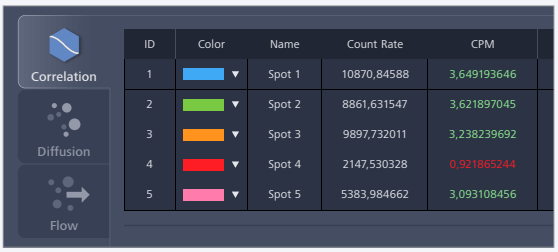


Figure 5 *Dynamics Profiler provides up to three analysis tabs: Correlation: auto-correlation data and analysis. Diffusion: Asymmetric Diffusion analysis by pair correlation, including visualization by polar heatmap. Flow: cross-correlation to reveal flow movement including speed (µm/second) and direction.*

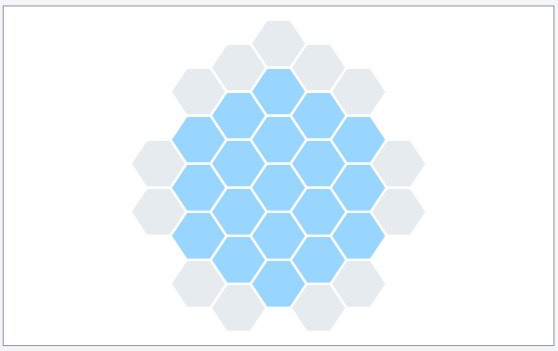


Figure 6 *This illustration depicts the highlighted 19 inner elements of the Airyscan detector used to calculate the autocorrelation (< 1.0 AU).*

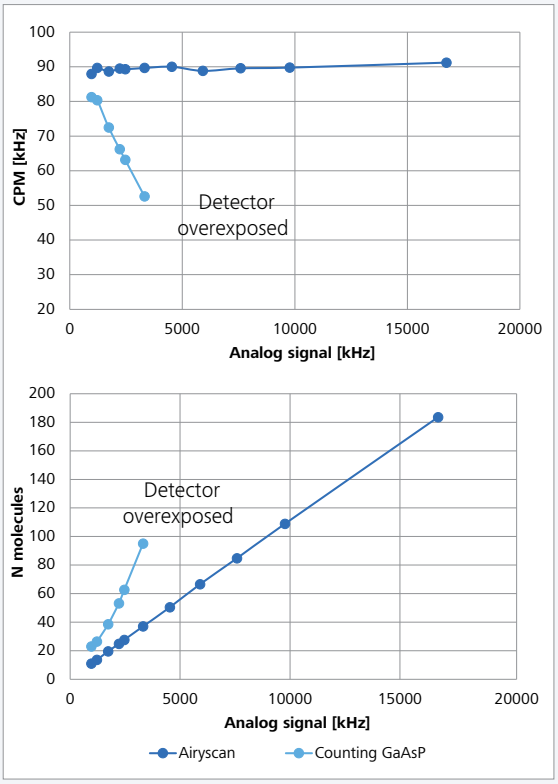


Figure 7 *Counting GaAsP detector(ChS LSM 980) vs. Airyscan detector (Dynamics Profiler)*

Dynamics Profiler (Airyscan detector) and classical FCS (GaAsP detector in counting mode; ChS, LSM 980) were compared using a dilution series of rhodamine 110. High dye concentrations led to the GaAsP detector being switched off. In contrast to the GaAsP detector, counts per molecules (CPM) were stable for the Airyscan detector along the entire dilution series and the number of molecules was reliably determined.

A) Correlation Tab:

The correlation tab is the first analysis and information presented after a Dynamics Profiler measurement is concluded. It provides data about molecular concentration and diffusion coefficient, based on the FCS principle as described in Box 1. The intensity trace data of the Airyscan's innermost 19 detector elements is joined (Figure 6). Unlike most conventionally used FCS detectors, Airyscan collects the intensity trace in analog mode and not in photon-counting mode.

The combination of analog mode and the read-out of 19 separate detectors, which splits the total signal and intensity changes, permits measurements at much higher total intensities (brightness) than conventional FCS would allow (Figure 7). Expression levels of fluorescent proteins are no longer a concern when planning or integrating molecular concentration and dynamics measurements in typical live imaging experiments.

B) Diffusion Tab:

Instead of the previously defined autocorrelation (Box 1), here the intensity traces of two individual detector elements are correlated with each other. This is called a pair correlation. The center element of the Airyscan detector is correlated with the Airyscan detector elements of the 3rd ring. The 3rd ring is divided into inner and outer elements (Figure 8), since even these small differences in distance to the central element will influence the correlation results. The individual correlations characterize the concentration and diffusion movement in 12 directions from the center and will highlight differences of these characteristics if present. Such differences in molecular behavior can be caused by cellular compartments, barriers, or molecular binding processes. While the spatial information of such regions can be deduced, this analysis does not provide information about the direction of movement. It is important that the center detector element of the Airyscan is positioned in one or the other compartment to clearly identify differences.

Within the software, the differences in diffusion and concentration are displayed with a color-coded circular diagram, the polar heatmap (Figure 9). The polar heatmap is derived from the respective correlation graphs of the individual pair correlations. The G(τ) values (see Box 1, FCS Principles) are color coded and within the polar heatmap the G (0) value starts in the

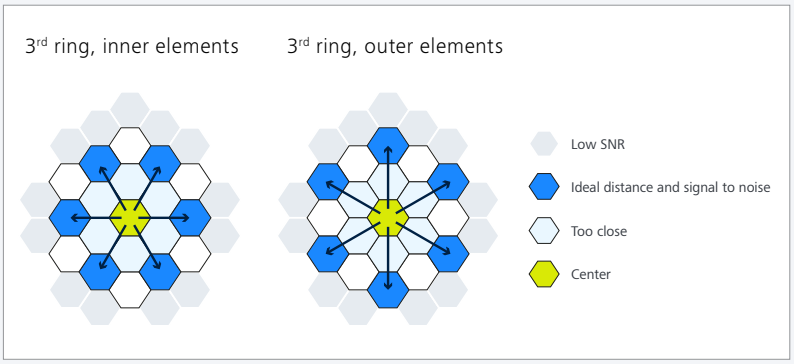


Figure 8 *Pair correlation*

This illustration depicts which detector pairs are used for pair correlation for the Asymmetric Diffusion analysis (Diffusion Tab), a cross-correlation of the middle detector element with individual elements of the 3rd Airyscan ring.

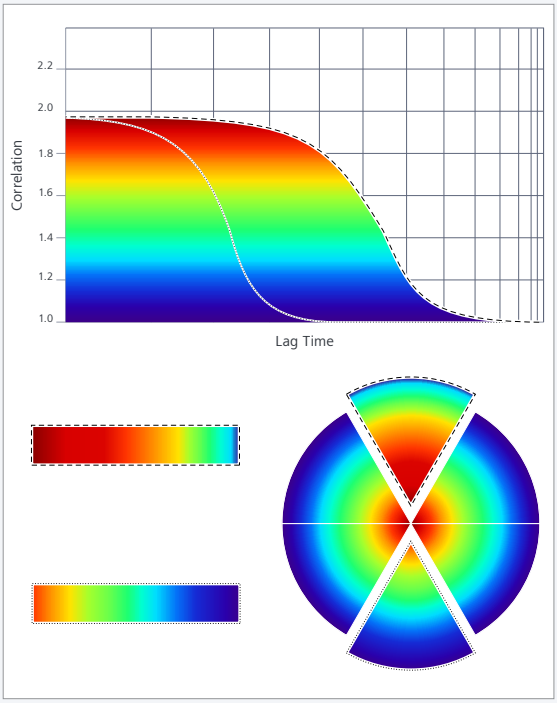


Figure 9 *Detecting heterogenous behavior with pair correlation*

This illustration depicts how the Pair Correlation Function (pCF) is color coded and used to display in a circular way, the polar heatmap. The individual pair correlations characterize the concentration and diffusion in 6 directions for each of the two polar heatmaps. The polar heatmap will highlight differences between correlation curves if present.

center of the circular diagram and continues by displaying the respective color gradient of the correlation graph. To better compare potential differences in diffusion time, the height of the correlation graphs can be normalized, omitting the concentration differences (see Appendix A for details).

C) Flow Tab:

To determine the flow direction and flow speed (µm/s) within a liquid, a total of 27 detector element pairs are cross-correlated (Figure 10) along 3 different axes along the Airyscan detector. Each of the three axes is defined by its line between two angles of the Airyscan detector, e.g., 30° ↔210°, with 0° ↔ 180° being defined as the vertical direction respective to the reference image, along each axis nine detector pairs are cross-correlated in both directions. Flow is detected if the cross-correlation graphs of the opposite directions differ. This can be easily seen when comparing the correlation graphs of both directions along the axis (Figure 11). The analysis determines if there is active molecular movement in either direction of this axis. Data of all axes combined, calculate the direction and speed of the molecules.

Raw Data: Collecting raw data of 32 individual detector elements and considering the seemingly endless possibilities to investigate molecular movement, all possible analysis options will never be available in a commercial software package. To ensure that individual and new questions can be addressed Dynamics Profiler provides all collected data to be used for individual and customized analysis. Detailed information on data export can be found in Appendix B.

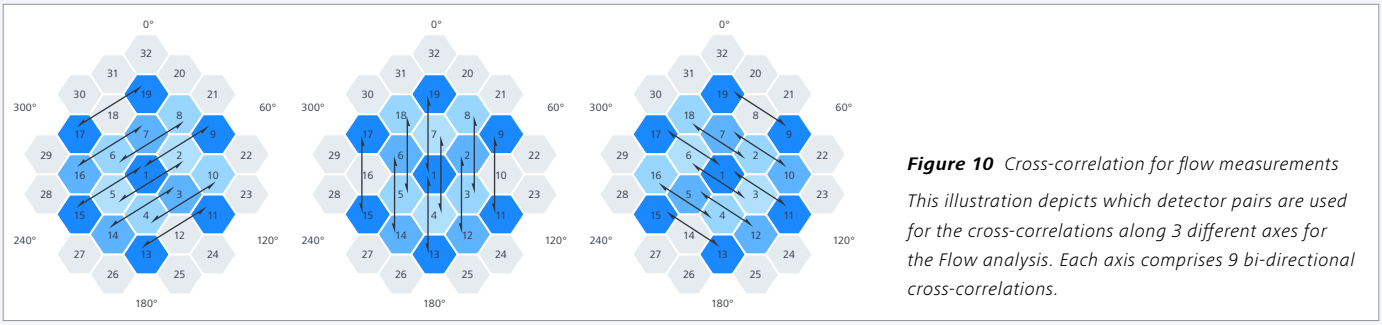


Figure 10 *Cross-correlation for flow measurements*

This illustration depicts which detector pairs are used for the cross-correlations along 3 different axes for the Flow analysis. Each axis comprises 9 bi-directional cross-correlations.

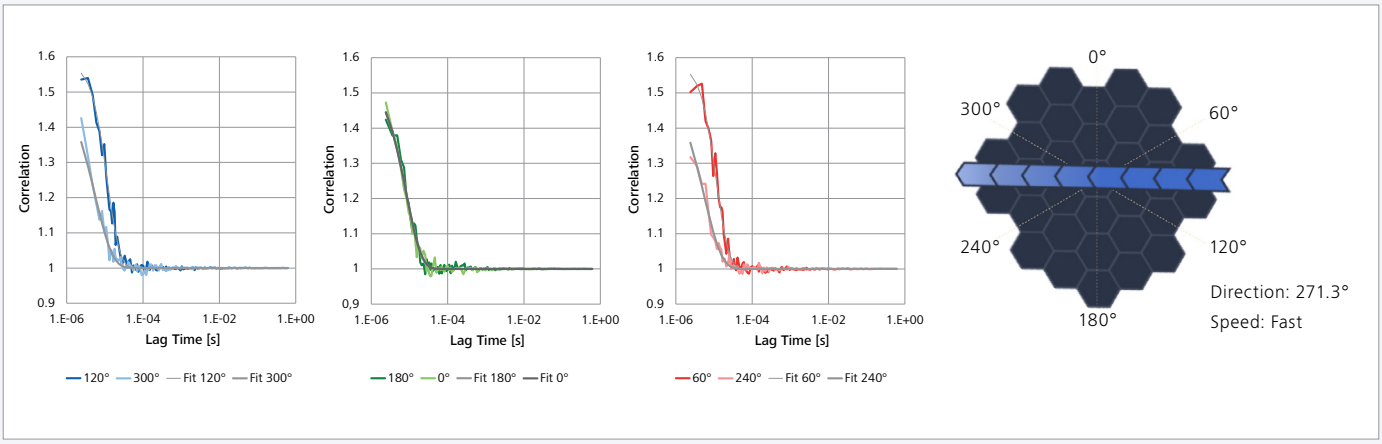
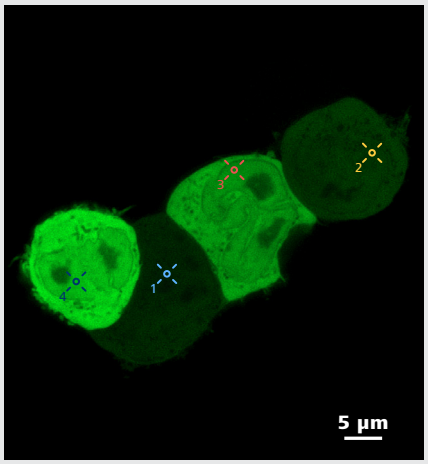


Figure 11 *Cross-correlation enables flow measurements.*

Flow speed and direction can be measured by cross-correlation of detector pairs (see Figure 9). The cross-correlation curves and their fits along three different axes are calculated (pairs of green, red and blue curves with fit in grey). If there is flow along one of the axes, it results in a shift to the right of the correlation curve (here 120° and 60°). Results of all three axes determine overall flow angle and flow speed in µm/s.



Freely diffusing eYFP in living cells

	Count Rate [kHz]	CPM [kHz]	Number of Molecules	Concentration [nM]
Spot 1	10826.0	3.9	2760.4	23074.1
Spot 2	12708.7	4.4	2887.6	24137.8
Spot 3	69683.2	5.3	13157.9	109988.1
Spot 4	101559.9	6.5	15663.6	130933.0

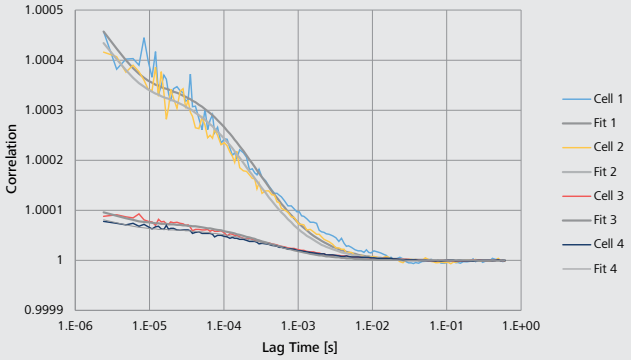
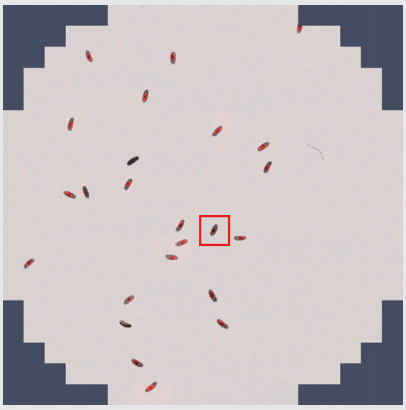


Figure 12 *Easy and fast access to dynamics and concentrations.*

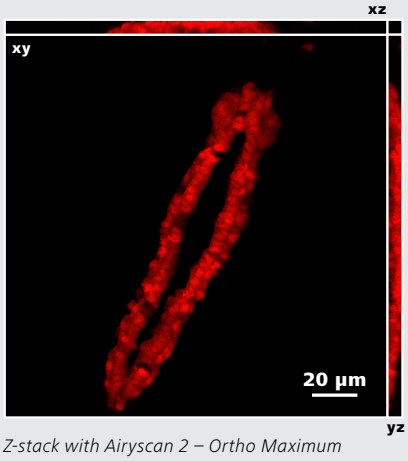
Dynamics Profiler allows easy and fast concentration measurements. Not only dim cells can be addressed but also bright cells can be included in the experiment. Sample courtesy of Peter O'Toole, Director of the Bioscience Technology Facility, Karen Hogg, Grant Calder, Graeme Park, Joanne Marrison, University of York, UK

For the correlation measurement, the mean signal of the 19 inner elements of the detector provides robust data, even for bright samples. During any live cell experiment, for example with a transgenic cell culture, the concentration of the expressed protein can be quickly determined and added to the characteristic of the observed and analyzed cells (Figure 12). Even though endogenous expression levels are desirable, more often the expression levels in cell culture or transgenic organisms are considerably higher. With Dynamics Profiler, expression levels of the fluorophore are of no concern and the measure-

Live cell experiment

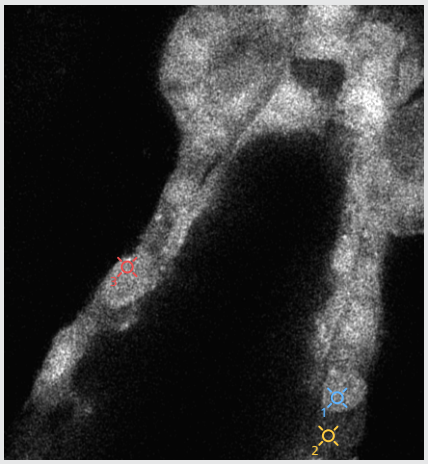


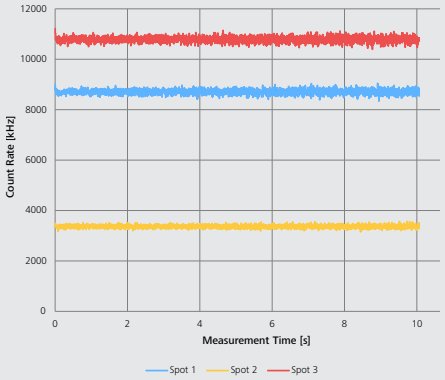
Overview scan



Z-stack with Airyscan 2 – Ortho Maximum Intenstiy Projection

Dynamics and concentration measurements





	Count Rate [kHz]	Concentration [nM]	Diffusion Coefficient [µm²/s]
Spot 1	8707.5	37263.1	242.9
Spot 2	3365.5	13666.4	279.4
Spot 3	10788.0	41997.8	239.9

Figure 13 *Add dynamics and concentration measurements to an imaging experiment.*

With the help of the Dynamics Profiler, more information can be easily gained in imaging experiments. These Drosophila melanogaster *embryos express mCherry under control of the hand enhancer in the embryonic heart. An overview scan allows for the identification of an embryo with the developmental stage of interest – further imaged with Airyscan to visualize the developing heart. Dynamics Profiler uniquely allows now additional measurements to effortless add dynamics and concentration to imaging experiments, even in brighter and thicker samples. Sample courtesy of Prof. Dr. Achim Paululat and Dr. Christian Meyer, Osnabrück University, Department of Zoology and Developmental Biology, Germany.*

ments can be added even at very bright samples (Figure 12, 13). Without changing the sample to adapt to low expression levels it is now possible to explore molecular dynamics. A variety of objective lenses, including long working distance options, makes it very easy to combine imaging data with reliable quantitative measurements of Dynamics Profiler.

Imaging data and quantitative dynamics measurements relate to pre- and post-experiment reference images of the sample. These images facilitate orientation and preserve the context of the measurement. The exact measurement area is depicted by a color-coded circle corresponding to the area of the detector (Figure 14). The exact position of the measurement area is particularly important when the experiment is going beyond a simple measurement of concentration or molecular dynamics but is looking for asymmetric diffusion characteristics. In this case a pair correlation (see Box 2) is conducted, uncovering heterogenous concentrations and behaviors of the protein of interest. These differences can be due to barriers within the cell, created by membranes or cell compartments. Changing behavior of proteins within and outside cellular condensates that form by liquid-liquid phase separation can be measured and their confined and asymmetric dynamic behavior analyzed. Previously investigating the formation of cellular condensates was hindered due to the high concentration of the molecules, making conventional FCS difficult to conduct. Dynamics Profiler solves this predicament by allowing these measurements to be conducted at very high molecular concentration and brightness levels, while adding spatial information at the same time (Figure 14).

Utilizing the additional data of the area detector gives a unique opportunity to measure liquid flow at a precise position in a volume. The direction and speed (in µm/s) of fluorescent proteins or dyes that are actively transported in a liquid can be determined in a single PSF volume. This way, the velocity of a liquid is measured at an exact point within the sample, and differences in flow can be recorded based on spatial position (Figure 15). For the first time, exact information can be collected that goes beyond average flow values based on volume per second or rough estimations by following vesicles or beads. The conditions for cells, organoids, or tissue culture, exposed to flow in microfluidic systems (e.g., organ-on-a-chip setups) based on moving liquid environments can be documented and be included in the experiment. Other fluids moved by e.g., heartbeat or other muscular movement can be characterized *in vivo*, by simply introducing fluorescently labelled proteins or dyes.

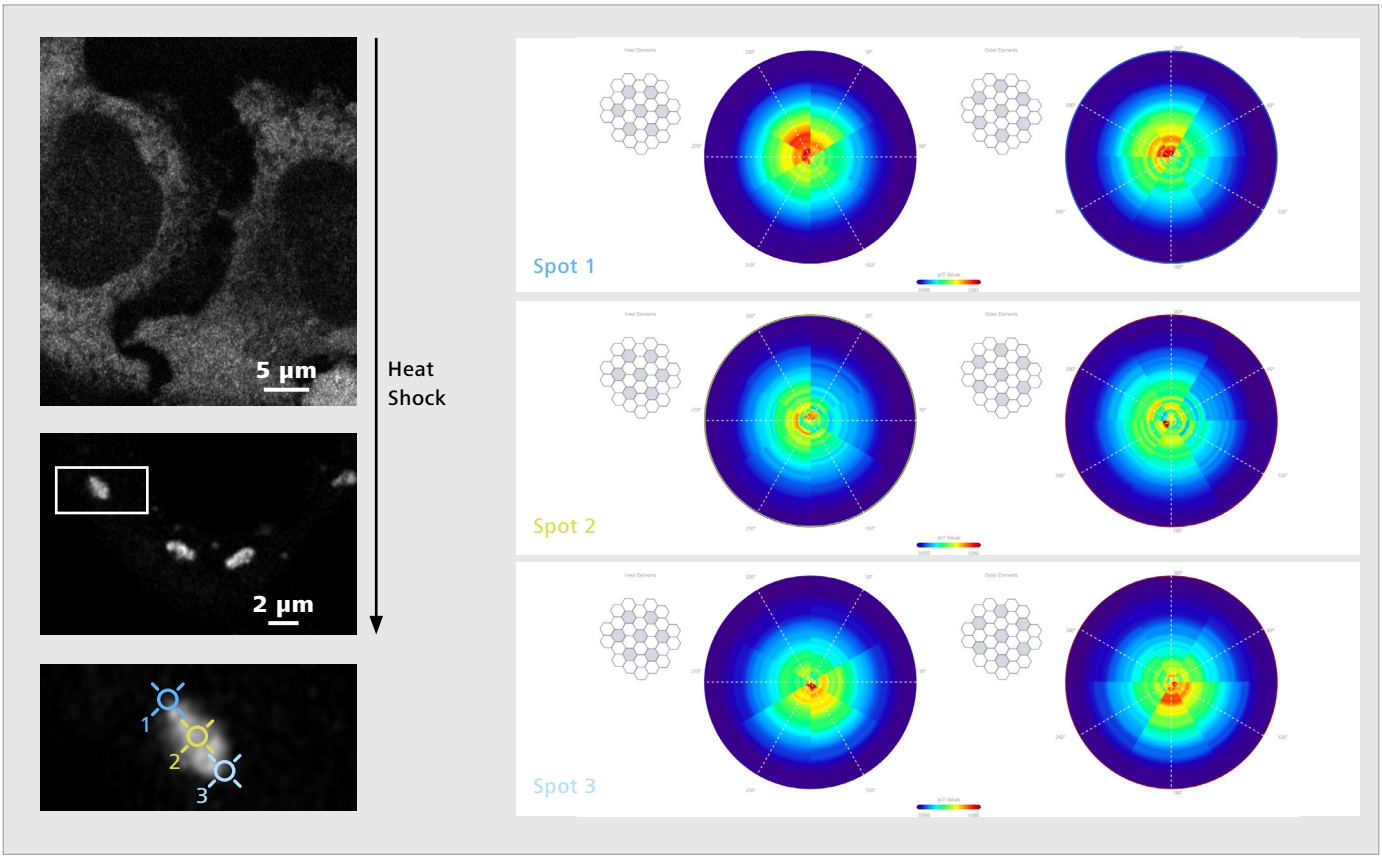


Figure 14 *Investigating dynamics during condensate formation.*

Spatial information collected allows to characterize heterogenous diffusion behavior, ideal to investigate cellular condensates that form by liquid-liquid phase separation. A reference image helps to position the spots and to orient within the sample. This example shows GFP-labeled stress granules in HeLa cells. Sample courtesy of Dr. V. Bader und Prof. Dr. K. Winklhofer, Institute of Biochemistry and Pathobiochemistry, Ruhr-University Bochum, Germany.

Comparison FCS (ZEISS) vs. Dynamics Profiler

Feature	ZEISS FCS	Dynamics Profiler
Reference image for orientation included	–	+
High concentration/bright sample	–	+
Pair correlation (Asymmetric Diffusion)	–	+
Flow measurement (speed, direction)	–	+
Wizard-guided	–	+
Accessible raw data	+	+
Include your own fit model	+	–*
FCCS	+	–
Spectral FCS up to 7 Channels	+	–
Diffusion coefficient and concentrations without additional calibrations	–	+
LSM 980	+	+
LSM 900 & LSM 800	–	+
Time resolution	66.7 ns	1.2 µs, ωr 0.5 µs

*Data can be exported for customized fit analysis

Workflow Dynamics Profiler

To ensure reliable and easily accessible dynamics data, the workflow of the Dynamics Profiler uses a wizard that has 3 main steps. The correct choice of hardware settings (e.g., suitable objective lens) is a prerequisite to enter the wizard – minimizing sources of error from the get-go. After starting the wizard, an automated Airyscan detector alignment is performed, ensuring the perfect measurement conditions and alignment precision for the upcoming experiment. Each dataset is therefore eligible for spatial Airyscan analysis. The second step allows the acquisition of a two-channel reference image in which the measurement spots will be defined in the subsequent third step of the wizard. Up to 10 measurement spots can be defined within the reference image, each spot being numbered and color-coded. The size of the spot represents the measured area. The field of view for Dynamics Profiler measurements might be smaller than the reference image, a gray border indicates out of scope areas into which no measurement spots can be placed. The spot icon harbors the information about the position of the spot and displays the corresponding area of the Airyscan detector that is used for data collection. This allows precise positioning of the measured area. The color of the spot icon can be changed to personal preferences and to optimize the contrast on the reference image. Prior to the measurement, each spot can be quality evaluated. To support the evaluation step, the Counts per Molecule (CPM) values are shown in a quality color-code: green = good, yellow = acceptable, red = insufficient. During the spot evaluation, the correction ring of the objective lens (if applicable), laser power, and the focus can be adjusted to maximize the CPM. Furthermore, the spot can be moved to optimize the position. The last step is to determine the duration of the measurement and afterwards start the experiment. Now all spots are measured for the set time in the previously defined sequence and concludes with the acquisition of a post-experiment reference image. The wizard automatically closes after experiment completion and the data along with the pre- and post-experiment reference images are displayed in the Correlation tab of the Dynamics Profiler. Up to three different analyzing tabs are available to view and analyze the data: Correlation Tab, Diffusion Tab and Flow Tab (see Box 2).

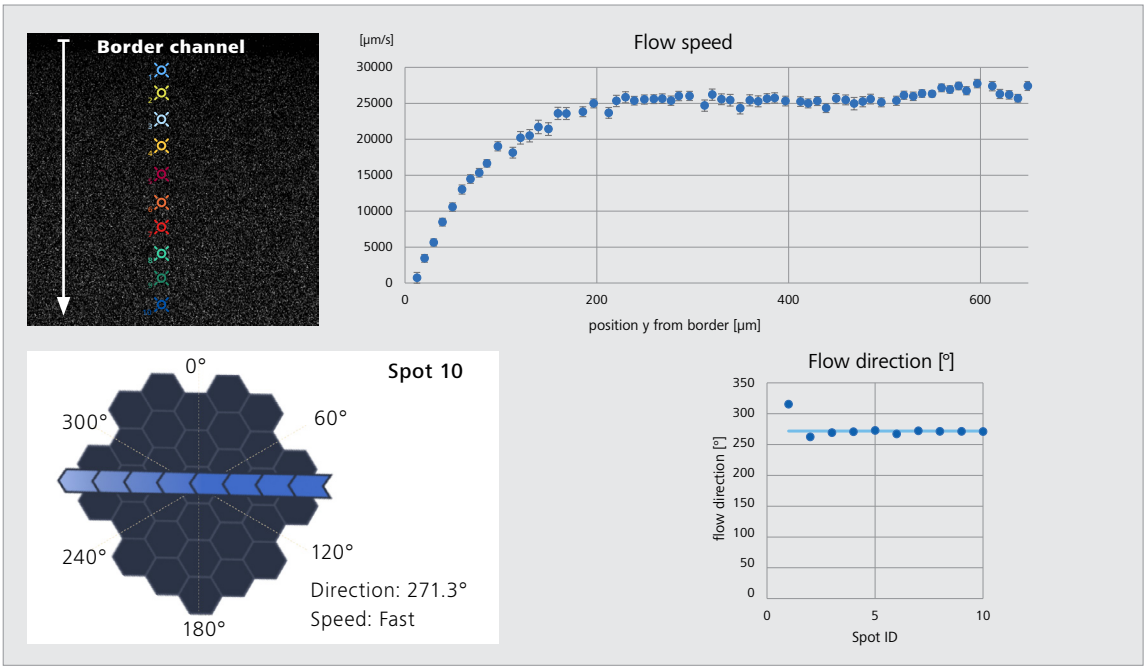


Figure 15 *Analysis of flow characteristics in a microfluidics channel*

Spatial information collected with ZEISS Airyscan enables flow measurements that provide unique new data related to microfluidics. Flow speed and direction of active movement can be determined for defined spots across a microfluidics channel. Laminar flow within such a channel can thus be characterized. A reference image helps to position the spots and to orient within the sample. Here, the Automated Sequential Injection System Aria by Fluigent was used to pump a rhodamine 110 solution through a microfluidic flow cell (1000 µm channel width).

Each step of the wizard independently saves the acquisition parameters. Entering the wizard for a second time restores all previous values in each step. Hence the subsequent measurements will be conducted even faster than the first.

Besides the default ωr calibration values, a custom ωr calibration is incorporated into the wizard; this calibration is optional and does not need to be done on a daily basis (see chapter Calibrations).

All data collected and their analysis result in a combination of images, tables, and graphical displays within the Dynamics Profiler. Each of these elements can be exported to be used for further data exploration, documentation, or publication. The values of any table can be exported into a text file when using the right mouse button anywhere in the table. All graphical elements can be saved as images (with or without background); and the pre- and post-experiment reference images can be saved as single .czi files.

For any further and future spatial correlation analysis, Dynamics Profiler saves the intensity trace data of all 32 detector elements (see Box 2 and Appendix B).

Similar to imaging, for Dynamics Profiler the better the acquired data, the better the result. In the case of Dynamics Pofiler the results are intensity traces and correlation curves. As best practice therefore, using the correct cover glass and immersion medium will go a long way to ensuring best data quality. As for imaging experiments, a high signal-to-noise (SNR) value will be beneficial to improve data. A good tool to estimate SNR are the color-coded CPM values. Finding a good compromise between laser power settings and measurement times is important for overall success. If the correlation curve is very noisy, it can be helpful to either enhance laser power or to increase the measurement times per spot.

Calibrations

Dynamics Profiler includes calibrations to ensure best performance. These calibrations are performed prior to or during installation by ZEISS personnel. The only exception is the optional "ωr Calibration Custom" which can be conducted by the user, if required.

Offset Calibration (dark noise correction)

The system's dark noise is largely due to "dark" noise of the electronic amplifiers. The maximum amplitude of this noise is much smaller than the amplitude of the signals generated by photons reaching the cathode of the GaAsP-PMT but could potentially influence the Dynamics Profiler measurement values for samples with low signal. To avoid this, the DP-offset (see below) is subtracted from the measured intensity trace signal (and values < 0 are set to 0). This ensures an almost complete elimination of dark noise signal within the measurement. The appreciably higher signal information is not significantly affected by this process. Subtracting a constant value from a time-resolved signal curve does not change the time correlation of the signals.

Prior to the Dynamics Profiler measurement, the dark noise is measured for ~13 ms. The Dynamics Profiler offset for each of the 32 Airyscan detector elements separately is calculated as:
DP-offset = mean value + 6 × standard deviation

Photon Conversion Factor

The Airyscan detector is an analogue detector so does not directly count the number of photons. Although the absolute number of photons is not needed to quantify FCS results (such as number of molecules or diffusion times), it is required to provide information on "count rate" and "counts per molecule". To do so, the analog GaAsP-PMT current (normally translating into the gray values in a picture) is converted into number of photons after suitable calibration.

Once determined for an individual GaAsP-PMT, the photon conversion factor does not change over time. This calibration is therefore performed only once at the ZEISS factory.

Optimal High Voltage Calibration

To ensure the best voltage setting (controlled by the Master Gain in ZEN) for the GaAsP-PMTs of the Airyscan detector during Dynamic Profiler measurements, this calibration finds the sweet spot for the detector high voltage setting. High voltage settings can cause dark noise spikes that should be avoided. Without any light source, the high voltage values of the GaAsP-PMT are varied stepwise and at each step the number of bright spikes is counted within a defined time frame. The optimal high voltage is the highest voltage value with less than 2 dark noise spikes within 30 s.

Because the dark noise behavior of the cooled and temperature stabilized Airyscan detector does not change over time, this value is calibrated once at the ZEISS factory.

ωr Calibration Default

Each Dynamics Profiler experiment provides the diffusion times and number of molecules within the measured excitation volume. To determine the respective diffusion coefficient and concentration, the actual radius of the PSF ωr is needed. The PSF is influenced by the LSM system itself, the used objective lens and the excitation and detection wavelengths.

Based on this information, a theoretical PSF diameter is provided and used for each experiment. The theoretical PSF differs from the acutal PSF of a specific system and objective lens, resulting in a typical deviation of up to ± 30% for diffusion coefficent

or concentration. This theoretical PSF is sufficient for most applications and avoids time consuming calibration tasks. Of course, the theoretical PSF cannot take unknown system and component tolerances into account. For a higher accuracy with a deviation down to ± 5%, Dynamics Profiler allows an optional custom ωr calibration (see next section) to conduct an exact radius measurement of the used PSF.

Custom ωr Calibration (optional)

If higher accuracy of the diffusion coefficient and the concentration value is needed, for example if measurements from different LSM systems are combined or compared, Dynamics Profiler provides the option to do an ωr calibration to determine the actual PSF diameter for the specific experiment configuration. It is therefore important that this custom ωr calibration is performed with exactly the same settings (e.g. laser wavelength, objective lens, emission filters) as the planned experiment.

The custom ωr calibration is included in the Dynamics Profiler workflow wizard. To start, a sample with a known diffusion coefficient (e.g., Rhodamine 110 dissolved in water) is chosen. In the correlation tab of the measurement, the ωr calibration view allows free entry of the diffusion coefficient or alternatively to choose the literature value from a preselected drop-down list of possible labels solved in water.

Now a one component FCS fit is performed, and the respective diffusion time determined. With the diffusion time now measured and the diffusion coefficient provided, the PSF diameter (ωr) is calculated. This value is saved in a customized named calibration file. Several of such custom ωr calibration files can be saved and used any time the respective experiments are conducted. The custom ωr calibration files will not overwrite the default ωr calibration values provided by the software.

Tips and Troubleshooting

Sample movement

When measuring molecular dynamics, it is important that the sample itself does not move and that larger particles do not interfere with the molecular dynamics. There are several methods to check for unwanted movement and some options to improve the measurement.

Check:

- in imaging mode, continuous or live scan, if there are components of the sample that are moving and could interfere with the molecular measurement, e.g. microtubule
- pre- and post-experiment reference images for any sample movement, especially when the measurement spot is positioned at a border or on a small sample
- your intensity trace measurement for large wave-like changes. If applicable use the "Add / Remove Region" tool to remove this part of the measurement or use the Dust Filter for small bright particles
- in the "Diffusion Tab" if the pair correlation curves are stacked on top of each other and all converge to 1. If not, this is a good sign that the sample has moved

To avoid measurement of sample movement and monitor movement better:

- place the spot in a larger area if possible
- keep the measurement time per spot low (10 seconds)
- use only 1 measurement spot per experiment, this helps to monitor better if something has moved or not
- refresh the reference image just before starting the experiment

Bleaching

Measuring a single spot for prolonged time can result in initial or continuous bleaching. Bleaching of fluorescent signal will influence the collected data.

- Be aware that immobile and slow particles will probably be bleached and are not represented in the dynamic measurement at all or not correctly
- If bleaching occurs, be aware that you might underestimate the number of molecules and concentration, even if you corrected the intensity trace with the Detrending function (see Detrending)

Signal to noise (SNR)

Like normal fluorescent imaging, high SNR values result in good data quality, which will influence the correlation graph and subsequent fit. In general, the same logic applies as for any imaging experiment:

- Collection of a sufficient number of photons is key, this translates here into optimal CPM
- Increasing laser power improves CPM, but as always keeping an eye on bleaching and keeping this to a minimum
- Increasing the measurement time improves the SNR, provided that sample movement is not a problem
- Ensuring that the optical beam path is optimal, e.g. cover slip thickness, immersion medium

Optical beam path

Similar to high-quality imaging, best results are achieved if the optical setup is optimal. When precise diffusion coefficient or molecular concentrations need to be achieved, the quality of the PSF is important and this is independent of whether the default or the custom ωr calibration is used (see chapter Calibration).

It is therefore important to check for each measurement:

- The setting of the objective correction collar (if available), with the sample in place
- The correct cover glass thickness
- Levelling of the cover glass / glass bottom
- Use of the correct immersion medium and correct embedding medium

Polar Heatmap (Diffusion Tab)

The polar heatmap of the Diffusion tab visualizes asymmetric diffusion characteristics within a measurement spot.

The performed pair correlation uses the single detector elements of the Airyscan detector, which leads to a lower SNR than for the correlation of the combined 19 elements for autocorrelation. In this case it may be necessary to improve the SNR with the above-mentioned tips, especially by increasing the measurement time. Furthermore, the "curve binning" option in the software can help to identify asymmetries.

Performing pair correlation over these short distances will lead to a certain amount of autocorrelation, so the measurement will be a combination of autocorrelation and pair correlation (cross-correlation). If the spot covers a region that lacks any of the protein of interest due to an impenetrable barrier, the autocorrelation will be dominant and will display faster diffusion in this area of the polar heatmap.

To identify regions of different molecular behavior, the center element of the Airyscan detector must be placed into one or the other compartment, not directly on the expected border between them.

If the border needs to be identified, it can help to place several spots along a line that crosses the expected border.

Flow Tab

The fit model for the Flow analysis is only suitable for molecular movement and therefore particles that are much smaller than the focal detection volume (roughly 100 nm or smaller). If larger particles are measured (e.g. large beads, cells) the flow analysis will still identify the direction; but the flow speed will most likely be underestimated.

If measurement of larger particles is needed, the collected raw data can be exported and used for customized fitting and analysis.

If the flow of the sample is too fast to fit the time resolution, the flow cannot be determined, and the software will show the "No Flow" graphics. To improve time resolution, repeat the measurement in ωr acquisition mode of the wizard.

Detrending

Before using the detrending function, ensure that the intensity trace measurement has been checked for any irregularities and signs of sample movement. Detrending should only be used to counteract the effect of bleaching.

Choose a Detrending value that results in a correlation graph that converges towards 1. If Detrending is too strong, the curve will swing below 1 at its end, and the Detrending value should be set to a higher number. The selected detrending parameter [ms] should be significantly higher than the diffusion time.

When bleaching occurs, the amount of labelled protein is decreased. If Detrending is used, the number of molecules will be a mixture of the bleached and non-bleached molecules and will most likely underestimate the number of molecules. If the part of the intensity trace is removed by the "Add cut region" function, only the remaining molecules are analyzed and again the number of molecules will be underestimated.

With a good Detrending setting, which is considerably higher than the diffusion time, the latter is not altered and can be analyzed.

Dust Filter

If larger bright particles interfere with the molecular measurement, resulting in occasional bright data spots in the intensity trace, the Dust Filter function can be used to select and remove these interfering signals.

Airyscan Alignment

The Airyscan alignment is an automated step of the Dynamics Profiler wizard which ensures the enhanced accuracy of the beam path for the measurements. To speed up the alignment step when initially starting the Dynamics Profiler for an experiment session, the normal imaging Airyscan alignment for the Airyscan track can be performed in Continuous or Live mode and the alignment position saved. When entering the Dynamics Profiler wizard, the Airyscan alignment starts from an almost optimal position and will take less time. The Airyscan alignment position is recalled if the Dynamics Profiler wizard is used multiple times in a sequence.

Vibrations

The measurement of intensity traces is sensitive to any movement of the molecules and unfortunately to any other movement as well. If the correlation graph shows oscillation, environmental mechanical vibrations or ambient light should be checked. Light sources like large computer screens or ceiling lightning can generate certain frequencies. Motorized elements placed on the system table can generate vibrations. The system table should also be checked to ensure it is correctly damped and attached to compressed air.

APPENDIX A

Calculation of the autocorrelation curve

Autocorrelation curve is calculated according to the formula [1]:

$$G(\tau) = \frac{< I(t) \cdot I(t+\tau) >}{(< I(t) >)^2}$$

The very first correlation value, corresponding to the time resolution of 1.2 µs, is skipped since it is biased due to correlation of the neighbor pixels after low pass filter due to digitalization.

Calculation of the correlation curve is done using an FFT-based algorithm (Fast Fourier Transform). A very small part of the signal at the end of the measurement (up to 0.5 %) might be disregarded to speed up the calculations.

Detrending Filter

The implemented FCS analysis (fit models) assumes that the measured data has no photobleaching effect. If photobleaching occurs during the measurement, the calculated correlation curves do not converge to one, and the corresponding data quality warning is displayed in the software.

The Detrending filter can be applied to remove this trend (e. g. due to the photobleaching) and restore the data for analysis; see as well "Detrending" in Tips and Troubleshooting. Detrended signal, $I_d(t)$ is calculated as follows:

$$I_d(t) = \frac{I_d(t)}{Tr(t)}$$

where the trend, $\boldsymbol{Tr(t)}$ is a gaussian smoothed signal:

$Tr(t) = I(t) \circledast e^{\frac{-t^2}{2\sigma^2}}$ with $\sigma = \frac{FilterWindowParameter}{4}$

FilterWindowParameter [ms] is set by the user. With the small parameter values, a high frequency trend is calculated. Selected detrending parameter should be much higher than the diffusion time, otherwise, very small artificial correlation values below 1 can be observed for the lag times close to the *FilterWindowParameter*.

Dust Filter

The Dust filter allows the removal of high intensity peaks (caused e. g. by aggregated objects) from the measured intensity trace. Calculations are done using the 500× down-sampled signal data. For comparison, intensity data shown in the count rate chart is down-sampled with factor 2000×.

Data bin is classified as dust (filtered out) if the following criteria is fulfilled: *UserParameter * I(t) > Mean(I(t))*

When both detrending and dust filters are activated, the dust filter is applied after the detrending filter.

Resulting cut regions are displayed in the intensity trace. A value for the dust filter should be selected that ensures only outlier intensity peaks are cut out.

Normalization of the correlation curves

Amplitude normalization of the correlation curves allows users to compare different correlation curves and see the differences in diffusion times.

If the fit of the correlation curves is already done, activation of the amplitude normalization is done using the amplitude fit parameter. Selected correlation curves are rescaled such that each selected curve has a correlation amplitude equal to 1 (corresponding number of molecules = 1).

$$G(\tau)_{norm} = \frac{G(\tau) - 1}{A} + 1$$

where A is a fitted amplitude.
In the case if no fit was done, correlation curves are normalized as follows:

$$G(\tau)_{norm} = \frac{G(\tau) - 1}{A_{mean}} + 1$$

where A_{mean} is the mean amplitude calculated over the first 10 points of the correlation curve:

$$A_{mean} = \frac{\sum_{i=1}^{10} G_i}{10} - 1$$

Fit

The unweighted fit of the correlation curve is done using Levenberg-Marquart algorithm.
Reduced chi2 is calculated as follows:

$$chi2 = \sum_{t=1}^{N} \frac{(G_t - F_t)^2}{(N - DF - 1)}$$

where G_t is the correlation curve values, F_t stands for fit values, N is the number of correlation / fit values, DF stands for the number of fit parameters.

Autocorrelation fit models

There are the following fit models available:
"One component 2D":

$$G(\tau) = A \cdot \left(1 + \frac{T_t \cdot e^{\frac{-\tau}{\tau_t}}}{1 - T_t}\right) \cdot \frac{1}{\left(1 + \frac{\tau}{\tau_{d1}}\right)} + 1$$

"One component 3D":

$$G(\tau) = A \cdot \left(1 + \frac{T_t \cdot e^{\frac{-\tau}{\tau_t}}}{1 - T_t}\right) \cdot \frac{1}{\left(1 + \frac{\tau}{\tau_{d1}}\right) \cdot \left(1 + \frac{\tau}{\tau_{d1}} \cdot \frac{1}{S^2}\right)^{0.5}} + 1$$

"Two component 2D" :

$$G(\tau) = A \cdot \left(1 + \frac{T_t \cdot e^{\frac{-\tau}{\tau_t}}}{1-T_t}\right) \cdot \left(\frac{cf1}{(1+\frac{\tau}{\tau_{d1}})} + \frac{1-cf1}{(1+\frac{\tau}{\tau_{d2}})}\right) + 1$$

"Two component 3D":

$$G(\tau) = A \cdot \left(1 + \frac{T_t \cdot e^{\frac{-\tau}{\tau_t}}}{1-T_t}\right) \cdot \left(\frac{cf1}{(1+\frac{\tau}{\tau_{d1}}) \cdot (1+\frac{\tau}{\tau_{d1}} \cdot \frac{1}{S^2})^{0.5}} + \frac{1-cf1}{(1+\frac{\tau}{\tau_{d2}}) \cdot (1+\frac{\tau}{\tau_{d2}} \cdot \frac{1}{S^2})^{0.5}}\right) + 1$$

"One component anomalous 2D":

$$G(\tau) = A \cdot \left(1 + \frac{T_t \cdot e^{\frac{-\tau}{\tau_t}}}{1-T_t}\right) \cdot \frac{1}{(1 + (\frac{\tau}{\tau_{d1}})^{\alpha})} + 1$$

"One component anomalous 3D":

$$G(\tau) = A \cdot \left(1 + \frac{T_t \cdot e^{\frac{-\tau}{\tau_t}}}{1-T_t}\right) \cdot \frac{1}{(1 + (\frac{\tau}{\tau_{d1}})^{\alpha}) \cdot (1 + (\frac{\tau}{\tau_{d1}})^{\alpha} \cdot \frac{1}{S^2})^{0.5}} + 1$$

where A is the correlation amplitude T_t is the triplet fraction τ_{d1} is the translational diffusion time S is the structural parameter τ_t=3µs is the fixed triplet relaxation time; $cf1$ is the first component fraction.

Data quality criteria

The correlation curve quality is considered good if the mean correlation value in the time range from 0.5 sec to 1 sec does not exceed 2 % of the correlation amplitude.
Data quality is also estimated using counts per molecule (CPM) values:

Data Quality	CPM value kHz/molecule
Red (insufficient)	smaller than 1
Yellow (acceptable)	between 1 and 3
Green (good)	higher than 3

Pair Correlation Function

Pair correlation function (pCF) is a set of cross-correlations between the central detector element and elements located at a given distance from the center (Figure 8).

For better SNR, cross-correlations are calculated in both directions (e.g. 1→8 and 8→1) and then averaged. Cross-correlation using the inner and outer elements of ring 3 (Figure 8, dark blue) provide most meaningful results.

pCFs are displayed as polar heatmaps. Quantitative interpretation of pCF is not implemented.

Flow analysis

To determine flow speed and direction, all possible cross-correlations are calculated at a distance of two fibers, within 3 inner detector element rings (Figure 10). All correlations in the same direction (for example, 5→2, 6→8, 17→19, etc.) are averaged, resulting in 6 correlation curves (by definition, 0° means vertical flow direction).

Flow fit models

All 6 curves are fitted globally by the following model:

$$G(\tau) = A \cdot exp\left(-\frac{r_0^2 + \tau^2 v^2 - 2 r_0 \tau v \cdot cos(\phi)}{4D\tau + w_r^2}\right) G_{diff}(\tau) + 1$$

where: v is the flow speed (fit parameter) ϕ is the angle between the flow and the vector connecting the fibers in this group of cross-correlations (fit parameter) r_0 is the apparent distance between the fibers (calibration parameter) $G_{diff}(\tau)$ is the diffusion term (2D or 3D), see Fit models. Other parameters are defined as in the "Autocorrelation fit models" section.

Flow speed classification

The calculated flow speed values are classified depending on the diffusion time of the analyzed fluorophore and based on statistical quality of the flow correlation curves:

Flow type	Criterium
No Flow	$v <= 3 * CI$
Slow	$(v > 3 * CI)$ && $(\tau_{flow} > 3 * \tau_d)$
Moderate	$(v > 3 * CI)$ && $(\tau_d < \tau_{flow} < 3 * \tau_d)$
Fast	$(v > 3 * CI)$ && $(\tau_{flow} < \tau_d)$

where $\tau_{flow} = \frac{r_0}{v}$, CI is flow speed confidence interval.

Calculation of the concentration and diffusion coefficient

Diffusion coefficient, D, is calculated as follows [2]:

$$D = \frac{\omega_r^2}{4\tau_d}$$

Fluorophore concentration is given as [2]:
$c = \frac{<N>}{V_e N_A}$, where $< N >= \frac{1}{G(0)-1}$ is an average number of molecules, N_A is the Avogadro constant.
$V_e = \pi^{3/2} \omega_r^2 \omega_z = \pi^{3/2} S \omega_r^3$, where S is structural parameter.

APPENDIX B

Dynamics Profiler raw data layout

The data are saved in CZI format. Each data point represents the 8-bit analog signal of a single Airyscan detector element. For performance reasons, the data are grouped as 1024 × 256 "pixels" XY subblocks, as shown below

Subblock with StartT = 0

	x = 0..1023			
y = 0..255	t = 1	t = 2	…	t = 1024
	t = 1025	…	…	t = 2048
	…	…	…	…
	…	…	…	t = 1024*256

Subblock with StartT = 1

	x = 0..1023			
y = 0..255	t = 1024*256 + 1	t = 1024*256 + 2	…	t = 1024*257
	t = 1024*257 + 1	…	…	…
	…	…	…	…
	…	…	…	t = 1024*512

…

Different acquisition spots are saved as positions corresponding to the "S" dimension (for example, all data subblocks for the second acquisition spot have StartS = 1). The "H" dimension (0 to 31) corresponds to the individual Airyscan detector elements 1..32, arranged as shown in Figure 16.

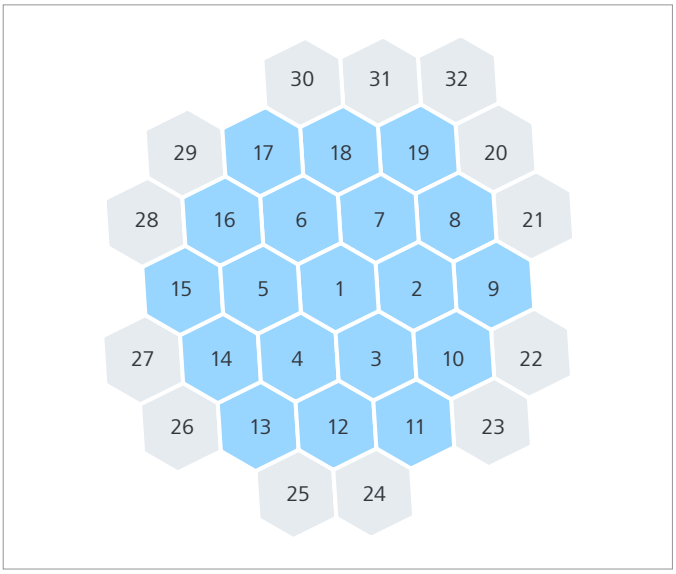


Figure 16 *Dynamics Profiler raw data layout*

Within the raw data, the individual Airyscan detector elements are saved in the H-dimension. Depending on the used LSM system (e.g. LSM 980 sideport versus LSM 980 rearport), the rotation of the Airyscan is following a matrix provided in the image metadata.

However, figure 16 does not consider detector rotation with respect to the image. The transformation to the image coordinates is system-specific and is defined by the matrix provided in the image metadata under the following path:

Information.Image.Dimensions.Channels[0].AiryScanSettings.Transformation, for example:

```
<AiryscanSettings>
    <TransformationXX>0.86162916044152571</TransformationXX>
    <TransformationXY>-0.50753836296070431</TransformationXY>
    <TransformationYX>-0.50753836296070431</TransformationYX>
    <TransformationYY>-0.86162916044152571</TransformationYY>
    …
</AiryscanSettings>
```

Calibration value of the Photon Conversion Factor is saved in the image metadata: Information.Image.Dimensions.Channels[0]:

```
<DetectorSettings>
    <PhotonConversionFactor>14.411915145986798</PhotonConversionFactor>
 </DetectorSettings>
```

For reading czi data in your application, we suggest to use the open-source libCZI library: GitHub - ZEISS/libczi: libCZI is an Open Source Cross-Platform C++ library to read and write CZI. Wrappers for Matlab (GitHub - ptahmose/MEXlibCZI: read CZI-documents from MATLAB) and Python (pylibCZIrw · PyPI) are also available.

Glossary

LSM	Laser Scanning Microscope
FCS	Fluorescence Correlation Spectroscopy
AU	Airy units
pCF	Pair correlation function
ωr	Effective radius of PSF
PSF	Point spread function
Intensity trace	time trace, count rate trace
FRAP	Fluorescence Recovery after Photobleaching
Cellular condensates	Biomolecular condensates, micron-scale compartments in eukaryotic cells that lack surrounding membranes but function to concentrate proteins and nucleic acids [3]
ROI	Region of interest
τ	Lag time
CPM	Counts per molecule
FFT	Fast Fourier Transform

References

[1] Comprehensive correlation analysis for super-resolution dynamic fingerprinting of cellular compartments using the ZEISS Airyscan detector, L. Scipioni, L. Lanzanó, A. Diaspro & E. Gratton, NATURE COMMUNICATIONS | (2018) 9:5120 | DOI: 10.1038/s41467-018-07513-2

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