**ConfoCor 2**

**Versatility is the Name of the Game**

**ConfoCor 2 analyzes in a single measurement**

- Diffusions,
- Interactions,
- Localizations,
- Molecule concentrations.

**ConfoCor 2 measures precisely and quickly**

- Highly sensitive (single molecules can also be measured)
- Short measuring times (within seconds).

**Cell biology meets biochemistry**

Measurement
- is non-invasive,
- runs under physiological conditions.

This means that measurements in the cell are as efficient as in solution.

**What is Fluorescence Correlation Spectroscopy?**
**Enhancing Time-tested Excellence**

**The strategy**

In 1972, the analytical concept of fluorescence correlation spectroscopy was developed by Elson, Magde and Webb, and Ehrenberg and Rigler enabled the dynamics of chemical equilibrium reactions and molecular diffusion processes to be examined. For the past 10 years, the technique has experienced an upswing after software, optical and detection problems had been solved reproducibly and at low costs.

**The design**

The technical implementation of the FCS design is based on the confocal detection of a fluorescence-labeled specimen excited with laser beams. The parallel beam of the excitation light is deflected via a dichroic beam splitter and focused on the specimen through an objective (Fig. 1). This focus is then imaged on a pinhole via the same objective and further optical components. The pinhole acts like a spatial filter. Only light from the focus can pass the pinhole unhindered, while light from other specimen areas is blocked.

Therefore, the benefit of confocal imaging lies in the fact that the detector only records light from a small, definable volume of the specimen. This detection volume measures less than $10^{-15}$ liters, which corresponds to the same order of magnitude as an *Escherichia coli* bacterium.

**The principle**

Fluorescence correlation spectroscopy is based on the measurement of the diffusion speed which is dependent on the molecule size. Fluorescence-marked molecules diffuse in a solution or a cell in accordance with the Brownian molecular movement. If a molecule passes through the confocal detection volume positioned in the waist (i.e. the focal point) of the excitation beam, a shower of fluorescence photons will be emitted (Fig. 2). These photons are gathered by the optics and directed to a sensitive single photon counter (APD).
The measurement

The detection of a fluorescence-marked molecule is performed in a volume which is limited by diffraction. Fig. 2 demonstrates the diffusion of an unbound dye molecule (small sphere marked in red) and a bound reaction partner (blue sphere) through the illumination focus. The black lines indicate the diffusion path. If a fluorescence-marked molecule is contained inside the detection volume, the emitted fluorescence photons can reach the detector. On account of the Brownian molecular movement, the number of fluorescent molecules entering and leaving the detection volume fluctuates incessantly. Due to the accidental nature of the described process, the measured fluorescence signal fluctuations must be statistically evaluated. This requires a sufficient number of molecule passages and photons per passage. As few as one hundred molecule passages are sufficient to permit the determination of the characteristic diffusion time of the specimen molecules and their number in the observation volume.

The statistical analysis

A software correlator analyzes the photons registered by the detector for time interrelations. A special statistical technique - called autocorrelation analysis - is used to gain characteristic numerical values from the measured signal, e.g. the average time required by a molecule to cross the detection volume and the average number of molecules simultaneously contained in the detection volume.

The autocorrelation function for an ellipsoid-shaped detection volume with Gaussian intensity distribution is:

\[
G(\tau) = 1 + \frac{1}{N} \cdot \frac{1}{1 + \frac{\tau^2}{\tau_D^2}} \cdot \sqrt{1 + \frac{\tau^2}{S^2} \cdot \frac{\tau_D^2}{\omega_1^2}}
\]

\[
N = \text{average number of molecules in the detection volume}
\]
\[
\tau = \text{correlation time}
\]
\[
\tau_D = \text{characteristic diffusion time}
\]
\[
S = \omega_2/\omega_1 \text{ Structure parameters}
\]
\[
\omega_2 = \text{radius of the detection volume}
\]
\[
\omega_1 = \text{radius of the detection volume perpendicular to the beam direction}
\]
Visualizing the Invisible

**Determining relevant parameters**

The correlation curve permits the direct reading of two parameters:
1. The point of inflection of the correlation curve permits reading of the characteristic diffusion time $\tau$ of the molecules in the detection volume (Fig. 3 A).
2. The height of the amplitude in relation to point $\tau = 0$ permits reading of the number $N$ of molecules contained in the detection volume on the average (Fig. 3 B).

This method enables various molecule species and their proportion in the total number of molecules to be determined in a single measurement. For this, however, the difference in the molecular weights $M_r$ must be large enough.

**Calculating relevant parameters**

Although the characteristic diffusion times are also dependent on the exterior shape of the molecule species, a direct relation between size and time can be described.

The characteristic diffusion time $\tau$ of the molecule through the volume element can be estimated using the diffusion constant $D$ or the diameter of the volume element $2\omega$.

Since the diffusion constant is dependent on the hydrodynamic radius $r$ of the observed molecule, and this – in turn – depends on weight $M_r$, we can express this in simpler terms: the characteristic diffusion time of comparable molecule densities is proportional to the third root of the molecular weight.

$$\tau_{\text{diff}} \sim \sqrt[3]{M_r}$$

$k$ = Boltzmann’s constant $1.38 \times 10^{-23}$ J/K
$\tau$ = Absolute temperature
$\eta$ = Viscosity
$N_A$ = Loschmidt number $6.023 \times 10^{23}$ mol$^{-1}$
$\rho$ = Average density of the molecule
$M_r$ = Molecular weight

**Fig. 3A**
The characteristic diffusion time is obtained from the value of the x-axis belonging to the inversion point of the correlation curve. The bigger a molecule, the slower it diffuses through the confocal detection volume. This is recognized by a shift of the autocorrelation curve towards higher correlation times.

**Fig. 3B**
The average number of particles in the detection volume can be calculated from the intersection point of the correlation curve with the y-axis. This shows that the correlation of the number of molecules is inversely proportional, i.e. the correlation values decrease when the concentration of particles increases.
The autocorrelation

In binding experiments, the smaller reaction partner is marked with a fluorescence dye. If binding exists between the now fluorescence-marked partner and another target molecule, the characteristic diffusion time will change. Fig. 4 shows an example of such a reaction.

Example of a molecule interaction

Fig. 4A
The picture shows an autocorrelation curve of a fluorescence-dyed antibody freely diffusing in the examination solution. The diffusion speed is $\tau_D = 1\text{ ms}$.

Fig. 4B
The presence of the antigen, an E. coli bacterium in the case of Fig. 4 B, causes an antigen-antibody reaction, causing a further, slowly diffusing component to appear with a diffusion speed of $\tau_D = 200\text{ ms}$. The autocorrelation curve permits the exact portion of the antigen-antibody complex to be detected in the examination medium.

TRITC = Tetramethylrhodaminisothiocyanat

$\tau_D$ = Antibody (1 ms)

$\tau_D$ = Antigen-Antibody-Complex (200 ms)

Specimen with two molecules marked in different colors

The cross correlation

If the molecules are not markedly different in size, the signal portions of the single components can no longer be reliably determined. Protein-protein interactions are an example of such an application with similar molecular weights. In this case, the cross correlation technique must be applied instead of the autocorrelation technique. The molecules are marked with two different dyes. The confocal volume element is illuminated with two wavelengths adapted to these dyes, and the signals are registered and correlated in two detection channels which are independent of each other (Fig. 5).

A software filter also cross-correlates the signals of the various channels, permitting the concentration of the bound molecules to be determined. The concentration values obtained allow the easy determination of the binding constant $K$ of the reaction.

$K = \frac{[\cdot]}{[\cdot\cdot]}$
Function and Structure

On account of the tiny size of the confocal measuring volume and its natural character, it is possible to place it in any position in the specimen that can be reached using the laser. In practice, it is possible to position the measuring volume inside a living cell, on its membrane or anywhere outside the cell.

Fig. 6
Taking measurements in the living cell
Biochemical processes determine the structures and functions of cells. With the combination of a confocal laser scanning microscope and a fluorescence correlation spectrometer, structures can be made visible and, at the same time, be investigated biochemically.
Precise spatial resolution is of major importance for *in vivo* experiments in particular. When the FCS module (ConfoCor 2) and a Laser Scanning Microscope (LSM 510) from Carl Zeiss are combined, any required number of measuring points within a cell can be marked three-dimensionally and evaluated within only a few seconds.

*Fig. 7 left*  
The ConfoCor 2 as stand-alone unit on the Axiovert 200. This configuration is ideal for measurements in solution and therefore for use in biochemistry.

*Fig. 7 right*  
Combination with the LSM 510 is the ideal choice for measurements in the cell.
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