

ConfoCor2-LSM 510

Detection of Protein Complex Formation

The present study demonstrates that multiprotein complex formation by the retinoblastoma tumor suppressor (RB) can be detected in the nucleus of living cells by fluorescence correlation spectroscopy (FCS) using the ConfoCor2 - LSM 510. FCS can be applied as a sensitive method for real time measurements of diffusion times for eGFP-fusion proteins in mammalian cells.

These characteristic diffusion times enable the calculation of diffusion coefficients and the molecular weight of complex assemblies to be determined.

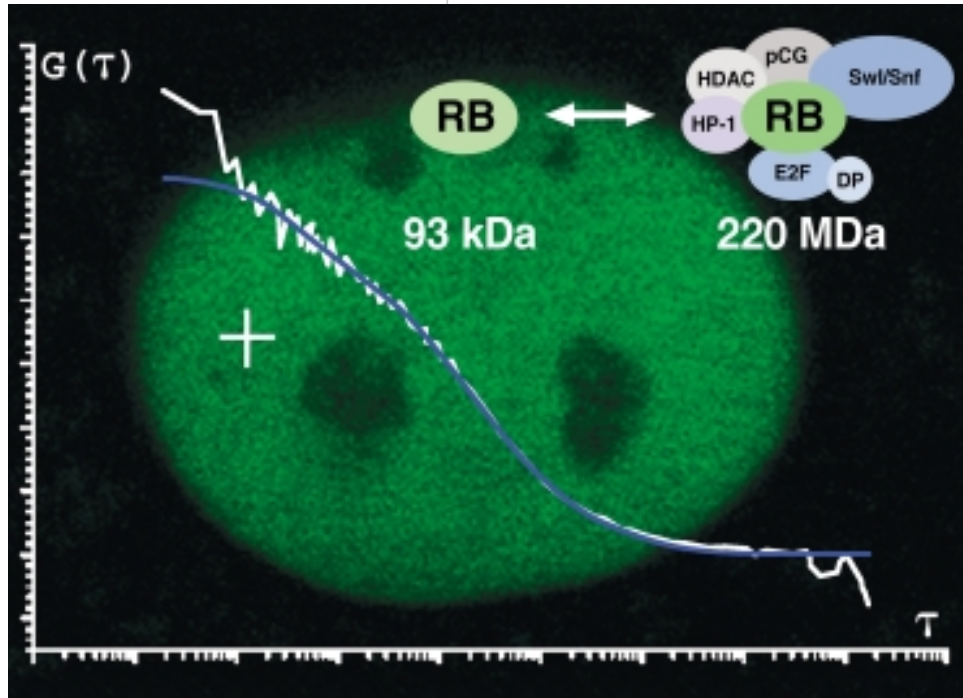
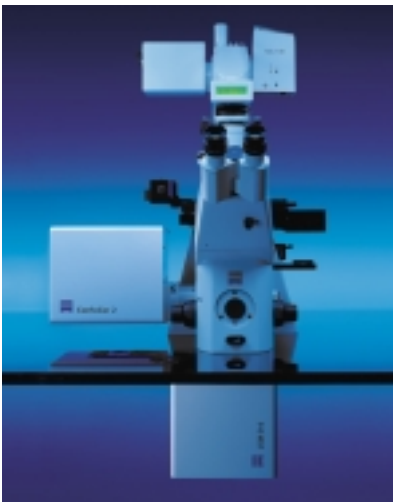


Fig.1 Detection of complex formation of eGFP-RB by FCS measurements within the nucleus of a living cell.



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**Fluorescence Correlation Microscopy
 within Living Cells**



Introduction

The retinoblastoma tumor suppressor (RB) functions as a negative regulator of the cell cycle. It was first identified because of its genetic inactivation in retinoblastoma. RB assembles multi-protein complexes to mediate transcriptional repression, cell cycle inhibition, and tumor suppression. Many RB binding partners have been identified from *in vitro* experiments, but the dynamics of RB-assembled complexes in living cells have not been investigated.

Most conventional techniques for studying protein interaction involve cell lysis and protein extraction. This biochemical fractionation often disrupts large complexes that exist within a cell and makes the study of complexes held together by weak or transient interactions impossible.

A common method to study protein/protein interaction in living cells is fluorescence resonance energy transfer (FRET). This method requires the labeling of the two putative interaction partners with distinct fluorescent markers, so-called FRET pairs. However, FRET only yields information about the proximity, but not about the direct binding of this FRET pair. Furthermore, it does not provide information about the mobility and the size of multi-protein complexes.

Within the last years a new, powerful tool for studying protein/protein interactions within living cells has emerged: fluorescence correlation spectroscopy (FCS) realized in the ConfoCor2 System from Carl Zeiss. With this method the movement of single, fluorescently labeled molecules through a focused laser beam is observed. The data obtained allow calculation of the mobility and the size

of the molecules. Thus, binding to other, non-fluorescent particles or structures can be measured, directly and in real-time. Due to the high sensitivity of this method, measurements can be performed even with weakly stained cells. If eGFP is applied as marker, a rather low - and thus more physiological - expression level of the eGFP fusion protein can be used.

In this study, we have for the first time investigated complex formation by RB in the nucleus of living mammalian cells in real time by using FCS. For this purpose we have expressed and measured RB-eGFP in Rat-1 cells. As a control we have studied the movement of free eGFP within the nucleus.

Materials and Methods

Cell culture and transfection

Rat-1 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. Rat-1 cells were seeded on 25-mm coverslips and transfected with 4 µg of eGFP or eGFP-RB expression vector by FuGENE 6 (Roche) according to the manufacturer's protocol. Thirty-six to forty-eight hours after transfection, coverslips were transferred to live-cell imaging chambers (Atto).

Equipment

FCS measurements were performed using the ConfoCor2 - LSM 510, equipped with an C-Apochromat 40x 1.2 NA water immersion objective.

Measurement

All measurements were performed at room temperature. eGFP fluorescence in cell nuclei was excited with 488-nm light from an argon laser and detected through a pinhole with a diameter of 70 µm and a 505 nm bandpass filter.

Analysis

Autocorrelation curves were derived from fluctuations of fluorescence intensity measured over 4-second intervals. 20 measurements were taken per nucleus and the corresponding average correlation curve was exported to the Microcal Origin software. These curves were fitted to a two-component model of free diffusion in two dimensions to derive the translational diffusion time and number of molecules diffusing through the confocal volume with D_{fast} and D_{slow} .

Equations for the calculation of diffusion coefficients, hydrodynamic radii, nuclear viscosity, and molecular weights from τ_D values measured by FCS.

$$D = \frac{kT}{6\pi\eta r} = \frac{\omega_{xy}^2}{4\tau_D}$$

$$r = \sqrt[3]{\frac{3m}{4\pi\rho N_A}}$$

D = translational diffusion constant

ω_{xy} = radius of laser focus in *xy*-plane

k = Boltzmann's constant

T = temperature

r = hydrodynamic radius

η = nuclear viscosity

m = molecular weight

N_A = Avogadro's number

ρ = mean density of the molecule

(1.2 g/cm³ for a globular protein)

Results

Fig. 2 shows that both eGFP and eGFP-RB are expressed in Rat-1 cells. Whereas free eGFP can be found in the nucleus as well as in the cytosol, eGFP-RB is mainly localized in the nucleus.

FCS demonstrated that free eGFP diffuses exclusively as a single species in the nucleus of Rat-1 cells with a τ_D of 205 μ s. This allows the calculation of a diffusion coefficient of 41.4 μ m²/s for free eGFP. Using the molecular weight of 30 kDa, we determined the nuclear viscosity of Rat-1 cells to be 2.41 gm⁻¹s⁻¹.

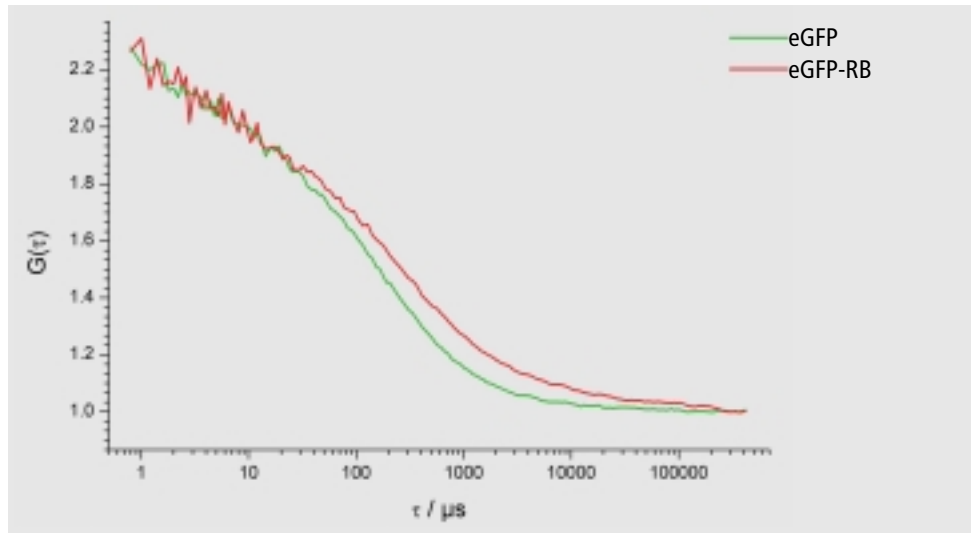
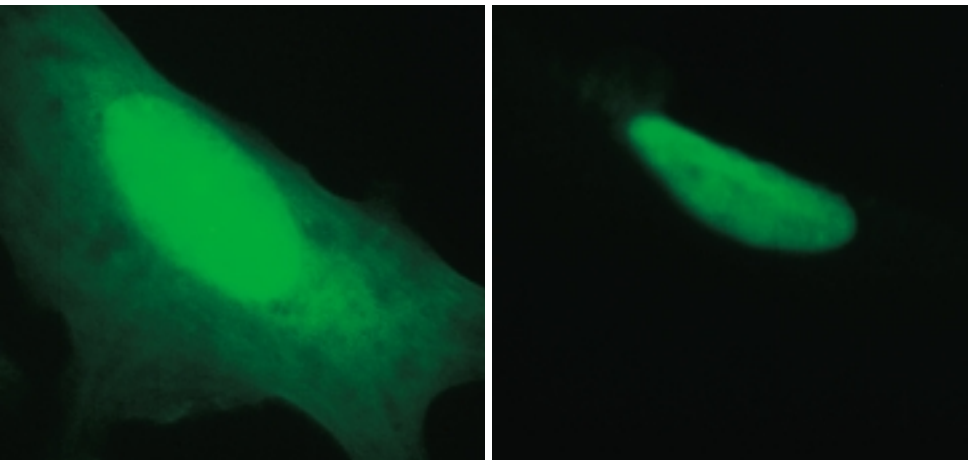


Fig.3 Normalized autocorrelation curves describing the behavior of eGFP (green) and eGFP-RB (red) in the nucleus of Rat-1 cells.

Fig.2 Expression of eGFP (left) and eGFP-RB (right) in Rat-1 cells.



A τ_D of 4000 μ s for the relatively immobile species corresponds to the assembly of a very large complex by eGFP-RB (greater than 200 MDa) in living cells.

Based on experiments performed *in vitro*, more than 100 proteins have been identified to associate with RB. However, no previous study has been able to examine the dynamics of RB-assembled complexes in the nucleus of living cells.

FCS revealed that eGFP-RB exists in two distinct states within the cell: the first as a highly mobile species with τ_D of 285 μ s, and the second as a large, relatively immobile complex with τ_D of 4000 μ s.

Using these τ_D values and the nuclear viscosity determined from free eGFP, we calculated that the rapidly diffusing species has a molecular weight of approximately 80 kDa. This is comparable to the predicted molecular weight of monomeric eGFP-RB (93 kDa) and suggests that this rapidly diffusing species is the monomeric form of the protein.

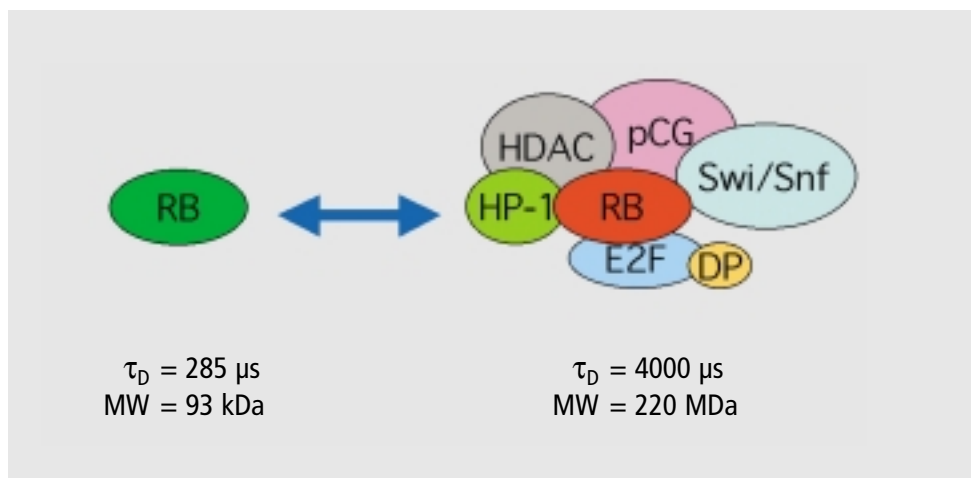


Fig.4 Data obtained from FCS analysis together with previously obtained biochemical results allows us to predict the following model for the dynamics of RB in the nucleus of living cells. (HDAC- histone deacetylase, HP1- heterochromatin protein 1, PCG – Polycomb group complex, SWI/SNF - SWI/SNF complex; E2F - RB associated proteins, DP – heterodimerization partner of E2F)



Conclusion

The ConfoCor2 - LSM 510 from Carl Zeiss enables both the study of labeled protein distribution within living cells and analysis of their mobility and binding to interaction partners. Using the ConfoCor2 - LSM 510 it is possible to define the spot of FCS measurement within the living cell in all 3 dimensions (x,y, and z).

In this study we have investigated a steady state complex formation of RB. We have shown that RB exists in an equilibrium between two species: a highly mobile, possibly monomeric state and a larger and less mobile complex. In addition, use of the ConfoCor2 - LSM 510 permits kinetic analyses of complex mobility on the order of minutes following a triggering event (e.g. pharmacological or chemotherapeutic agent).



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Subject to change.

Further reading

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