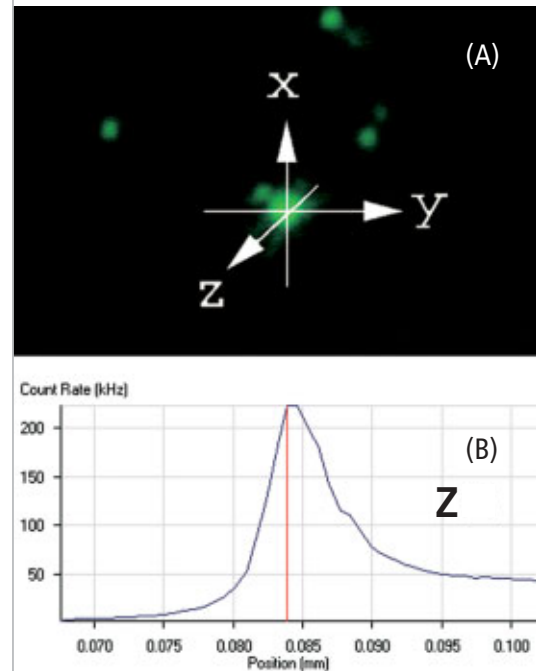


## Nuclear Dynamics of SP100

Recent developments in cell biology and microscopy techniques have made it possible to analyse proteins in their natural setting: the living cell. Live cell imaging of proteins tagged with a fluorescent marker (i.e. green fluorescent protein, GFP) have provided profound insights into how proteins interact and work in the living cell. Such observations have revealed that most of the proteins analysed so far are not bound statically to other cell components but show very dynamic behaviour.



Here, we have measured the dynamics of a nuclear body (NB) protein (SP100) using fluorescence correlation spectroscopy (FCS) in nuclei of living mammalian cells. In the nucleoplasm we find two fractions of SP100 with different mobilities: one fast fraction that appears to roam the cell nucleus in a random search for possible binding partners, and a slower moving pool that probably represents oligomeric forms of SP100. Within the nuclear body FCS reveals a further, very slow or immobile pool of SP100. This fraction may represent a storage form of the protein or a docking-site for other NB proteins. Altogether, FCS reveals at least three fractions of SP100 with different mobilities in the nuclei of living cells. These distinct mobilities are consistent with the proposed biochemical function of SP100. Strikingly, the kinetic behaviour of the SP100 nucleoplasmic pools were not accessible by standard bleaching techniques, demonstrating the power of FCS to analyse protein kinetics at resolutions far below the threshold of confocal microscopy.



**Fig. 1**  
Positioning the laser beam on a PML body  
(A) Cell nucleus of a cell expressing a GFP-tagged SP100.  
(B) Z-scan through the PML body and positioning of the laser beam.

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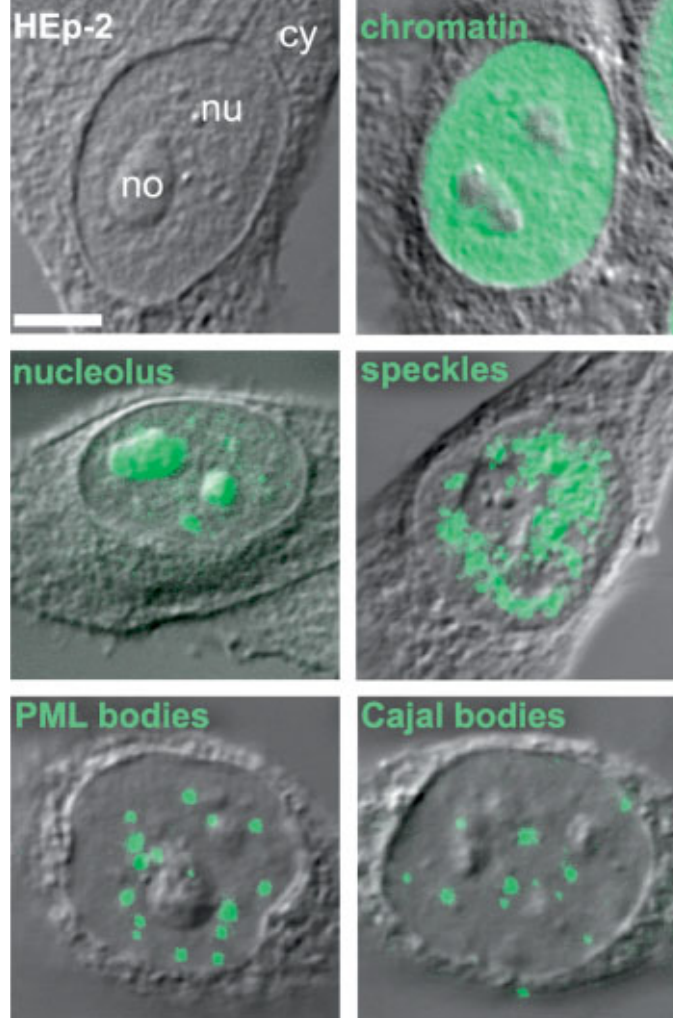
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**Discrimination of three SP100 populations  
Fluorescence Correlation Microscopy  
within Living Cells**



We make it visible.



**Fig. 2 Subnuclear structures**  
*Confocal microscopy images of HEP-2 cells stained with specific antibodies directed against the indicated nuclear substructures (green). Differential interference contrast (DIC) images (grey) were recorded from each cell at the same time and merged with the respective immunofluorescence image. DIC reveals the cytoplasm (cy), nucleoplasm (nu), and nucleoli (no) in HEP-2 cells (HEp-2). Subnuclear structures were detected with antibodies against histone H2A (chromatin), fibrillarin (nucleolus), promyelocytic leukemia (PML) protein (PML bodies), and p80 coilin (Cajal bodies). The bar represents 5  $\mu$ m.*  
 Images adopted from: von Mikecz and Hemmerich (2005) *Subnuclear pathology*; in: *Visions of the Cell Nucleus*, American Scientific Publishers, CA, USA, eds.: Stephan Diekmann and Peter Hemmerich.

## Introduction

A hallmark of the mammalian cell nucleus is the presence of visually defined structural compartments (Fig. 2). Most of these structures participate in the synthesis, processing and modification of RNA, and form in response to gene expression. Components of the ribosome biogenesis pathway are predominantly confined to the nucleolus, while proteins and ribonucleoprotein complexes involved in mRNA metabolism occupy the interchromatin space. Compartments identified in the interchromatin space include nuclear speckles containing spliceosomal components, Cajal bodies involved in snRNP biogenesis, and PML nuclear bodies enriched in proteins with a variety of functions (Fig. 2).

The definition of specific biochemical interactions among nuclear proteins in distinct compartments has led to a picture of structural continuity and functional stability within the nucleus. However, recent studies based on observing fluorescently tagged proteins within living cells indicate that the component parts of these structural entities are clearly in a state of flux. The structural instability of subnuclear compartments such as the nucleolus and nuclear bodies seems to confer functional flexibility, but this flexibility comes at a price: many nuclear proteins are known to interact dynamically with one or other of these compartments, but disruption of the specific organisation of nuclear

proteins can result in cell function defects and cause molecular-based disorders. In order to gain more insights into such subnuclear pathologies, it is important to study the mechanisms of nuclear body formation, maintenance and disassembly.

It has been suggested that nuclear bodies (NB) serve as depots for specific nuclear proteins, as active sites for biochemical reactions, or as recycling centres for inactive proteins or protein complexes. One way to address this open question is to determine the subnuclear dynamics of proteins present in NBs. In recent

years, bleaching techniques such as FRAP (fluorescence recovery after photobleaching) have been developed to study the kinetic behaviour of cellular proteins.

The recently developed combination of confocal microscopy with fluorescence correlation spectroscopy (FCS) has opened the door to directly investigate the kinetic properties of fluorescent proteins within femtoliter volumes in living cells. We have used this approach to study the dynamics of SP100, which is predominantly localised in distinct nuclear bodies, but is supposed to function at chromatin.

# ConfoCor 2 - LSM 510 META

## Results

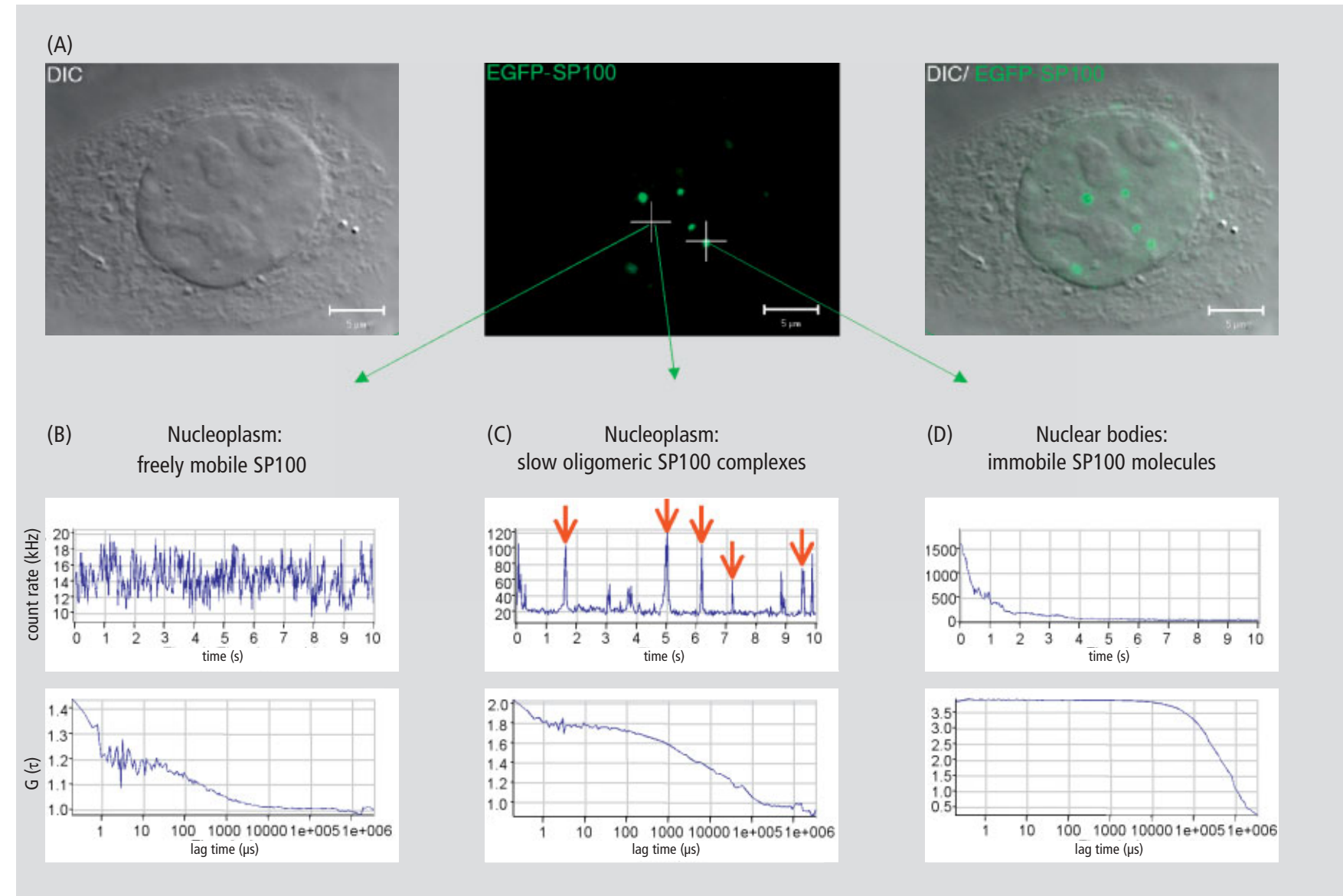
In order to measure the mobility of SP100 in the nucleoplasm, the FCS laser beam was positioned within the nucleus, adjacent to the nuclear bodies (Fig. 3A, middle panel). Note that GFP fluorescence was not detectable by confocal microscopy in those regions of the nucleus, indicating the low abundance of SP100 within this nuclear compartment. Due to this low fluorescence, classical bleaching techniques failed to monitor GFP-SP100 dynamics in the nucleoplasm (not shown). In contrast, SP100 protein kinetics were readily accessible by FCS measurements (Fig. 3B). FCS provided count rate traces giving rise to autocorrelation curves that could be fitted to an anomalous diffusion model (Fig. 3B, eq. 2). The diffusion coefficient of GFP-SP100, as calculated from this equation, was  $D=2.35 \mu\text{m}^2/\text{s}$ .

In some count rate traces several spikes were observed that dominate the correlation curve (Fig. 3C). These spikes correspond to large mobile structures containing several GFP-SP100 molecules. These oligomers migrate more slowly due to their increased size and increased binding to chromatin. They are more prone to bleaching than dimeric or monomeric molecules since they remain longer in the illuminated spot.

Autocorrelation curves obtained from recordings with infrequent fluorescence peaks (Fig. 3C) can be fitted to eq. 2 and the diffusion coefficient

for this population of SP100 was  $D=0.12 \text{ cm}^2 \text{ s}^{-1}$ . Thus, the large SP100 complexes are ca. 6-fold slower in the nucleoplasm than the freely mobile SP100 population.

Next, we investigated the mobility of SP100 within nuclear bodies. The FCS laser beam was positioned within the brightly stained structures (Fig. 3A, middle panel) and count rate traces were recorded (Fig. 3D). The initial fluctuation decay within the first seconds is indicative of an immobile or very slow-moving fraction of SP100 within the nuclear body structure. Confocal imaging of cells within the nuclear bodies, after FCS, indeed revealed that these no longer fluoresce (not shown), confirming the presence of an immobile SP100 population. After this initial "bleaching" period the recorded count rate trace resulted in autocorrelation curves similar to those obtained for SP100 in the nucleoplasm. Consequently, the diffusion coefficient determined was the same as for the freely mobile SP100 population (Fig. 3D).



**Fig. 3**  
 Three populations of SP100 with different mobilities in the nucleus of a living cell.  
 (A) Confocal image of an HEp-2 cell expressing GFP-tagged nuclear body protein (GFP-SP100). A differential interference contrast image (left) and the GFP fluorescence (middle) of the same cell were recorded separately before the FCS measurement. The image on the right shows the result of merging the two split images. Subsequently, FCS measurements were performed at different positions in the nucleus (crosses).  
 (B) and (C) show count rate traces and respective autocorrelation curves for FCS measurements within the nucleoplasm.  
 In (D) the FCS laser beam was positioned in x,y, and z to hit a nuclear body.

## Materials and Methods

### Cell culture and transfection

HEp-2 cells were grown on chambered coverglasses (Nunc) in a 10% CO<sub>2</sub> humidified atmosphere at 37°C in DMEM supplemented with 10% fetal calf serum. Cells were transfected with plasmids expressing a EGFP-SP100 fusion protein, using Roti-fect (Carl Roth) and stably expressing cells were selected by adding G418 to the medium.

### Equipment

FCS measurements were carried out on a ConfoCor2 – LSM 510 META, equipped with a C-Apochromat 40 x 1.2 NA water immersion lens.

### Measurements

All measurements were performed at 37°C. Molecules were excited with the 488 nm line of an argon ion laser and fluorescence was detected with a 505 nm long-pass filter. The tube current was set to 4.8 A and the AOTF to 0.1% to avoid sample bleaching. Images were acquired by laser scanning microscopy.

### Analysis

For every data point, a series of 10 subsequent autocorrelation measurements were carried out for 10 s each. A minimum of 10 cells were analysed. The averaged autocorrelation functions were fitted with the built-in routines of the ConfoCor2 software accounting for two diffusive components and a triplet transition. Alternatively, the correlation curves were exported to the Microcal Origin software and fitted with an algorithm accounting for anomalous diffusion.

### Equations:

Formula for free dimensional diffusion of two components (1) and anomalous diffusion of one component (2) with triplet fraction and calculation of diffusion (3) and transport (4) coefficients, as well as their relationship (5).

$$(1) \quad G(\tau) = \frac{1}{N} * \left(1 + \frac{T * e^{-\tau/\tau_T}}{1-T}\right) * \left( \frac{1-y}{\left(1 + \frac{\tau}{\tau_{D1}}\right) * \left(1 + \frac{\tau}{\tau_{D1} * S^2}\right)^{1/2}} + \frac{y}{\left(1 + \frac{\tau}{\tau_{D2}}\right) * \left(1 + \frac{\tau}{\tau_{D2} * S^2}\right)^{1/2}} \right)$$

$$(2) \quad G(\tau) = \frac{1}{N} * \left(1 + \frac{T * e^{-\tau/\tau_T}}{1-T}\right) * \left( \frac{1}{\left(1 - \left(\frac{\tau}{\tau_A}\right)^\alpha\right) * \left(1 + \left(\frac{\tau}{\tau_A}\right)^\alpha * \frac{1}{S^2}\right)^{1/2}} \right)$$

(3)

$$D = \frac{\omega_{xy}^2}{4 * \tau_D}$$

(4)

$$\Gamma = \frac{\omega_{xy}^2}{\tau_A^\alpha}$$

(5)

$$D(\tau_A) = \frac{\Gamma * \tau_A^{\alpha-1}}{4}$$

$G(\tau)$  = correlation function  
 $\tau$  = lag or correlation time  
 $N$  = number of total molecules  
 $T$  = fraction of triplet state  
 $\tau_D$  = free diffusion time  
 $\tau_A$  = anomalous diffusion time  
 $\tau_T$  = relaxation time of triplet state  
 $y$  = fraction of species 2  
 $1-y$  = fraction of species 1  
 $\alpha$  = anomalous diffusion exponent  
 $S$  = structural parameter  $S = w_z/w_{xy}$  ( $w$  equal the 1/e<sup>2</sup> intensity values of laser beam in axial (z) and radial (xy) directions)  
 $D$  = diffusion coefficient  
 $\Gamma$  = transport coefficient

## Conclusions

In this report we demonstrate the application of FCS to study diffusion processes in the nuclear compartment. Its high sensitivity allows measurements where other imaging-based technologies fail due to a low abundance of proteins. We demonstrate here that SP100 shows a distinct dynamic behaviour depending on its nuclear localisation. Such differences seem to be significant with respect to SP100 function in the organisation of chromatin. The freely mobile population and the oligomeric complexes most likely represent SP100 molecules actively involved in the chromatin compartment where they may function in transcriptional regulation, while the immobile or very slow fraction may be inactive by being sequestered into the nuclear bodies. Here, SP100 is likely to be stored or modified in a transient manner (very slow fraction) until released again into the nucleoplasm in a functional form.

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