Calculation of diffusion coefficients and diameters of dextran fractions from FCS measurements

To obtain diffusion coefficients from the autocorrelation function calculated from fluorescence correlation spectroscopy (FCS) measurements, we fitted the function below (Aragon and Pecora, 1976; Thompson, 1991; Wohland et al., 2001): 

$$G'(\tau) = 1 + \frac{1}{N} \cdot \frac{1}{1 + 4D\tau/\omega^2} \cdot \sqrt{\frac{1}{\eta + 4D\tau/(S\omega)^2}}.$$  \hspace{1cm} (1)

where: $G'(\tau)$ is the value of the autocorrelation function at time ($\tau$); $\tau$ is the correlation time; $\omega$ is the dimension of the confocal volume in XY direction (It was determined before the experiments for each laser line by using Rhodamine-6-G as standard [D = 280 $\mu$m$^2$ s$^{-1}$]); $S$ is the ratio between the Z and XY dimensions of the confocal volume (It was fixed to 5 according to the ConfoCor 2 user manual’s description.); $N$ is the number of particles in the confocal volume; and $D$ is diffusion coefficient.

$N$ and $D$ were fitted using the software Origin (OriginLab Corp.). For each dextran fraction ten measurements at three different laser intensities were made and such experiments were repeated twice. Diffusion coefficients shown in Table I are averages.

We also measured each dextran fraction labeled with different fluorophores. We found no change in the diffusion coefficient of a given dextran measured by FCS and the same molecular weight fraction showed exactly the same entry kinetics into oocyte nuclei after cytoplasmic injection independent of its fluorescent label (see Fig. S6). Thus, the labeling with different fluorophores did not affect the diffusional properties of dextrans. Oocytes injected with fluorescently labeled dextrans matured with normal timing and morphology.

The hydrodynamic radius ($R_{H}$) was calculated from the measured $D$ values using the Einstein equation. In turn this was converted to gyration radius ($R_{G} = 1.54 \times R_{H}$), which gives a better description of the molecular dimensions for random coils, such as dextrans (Luby-Phelps, 1989). This results in the equation below:

$$R_{G} = 1.54 \times \frac{kT}{6\pi\eta D}.$$  \hspace{1cm} (2)

where $k$ is the Boltzmann constant ($1.38 \times 10^{-23}$ J K$^{-1}$), $T$ is the temperature (293 K), $\eta$ is the viscosity of water (0.01 g cm$^{-1}$ s$^{-1}$), $D$ the diffusion coefficient, 1.54 is the ratio between the gyration and hydrodynamic radii for ideal random coils also valid for dextrans >2 kD (Luby-Phelps, 1989).

Calculated diameter (=$2 \times R_{G}$) values are shown in Table I.

Image acquisition and analysis

Imaging was done on a customized ZEISS LSM510 Axiocam confocal microscope equipped with a fast z-scanning stage (HRZ 200) and selected PMTs. Lasers were switched line-by-line in all experiments to avoid cross-talk and time shift between frames. In the dextran injection experiments, images were collected every 12 or 15 s (~30 s for GFP constructs). Time lapse was decreased to 5 s at the onset of phase II of NEBD. In these experiments a single optical section along the animal-vegetal axis was imaged. To visualize the permeabilization wave (see Fig. 6) z-stacks were collected as fast as possible (at 256 $\times$ 256 $\times$ 20 voxels every 4.5 s). 3D reconstructions of these sequences were done by manually segmenting the intact and permeabilized nuclear surface areas, the two resulting datasets were then rendered in Amira 2.3 (TGS, Inc.) as isosurfaces. Mean fluorescence intensities were measured in ROIs in the nucleus, cytoplasm, and for background outside the cell in the LSM510 software (Carl Zeiss MicroImaging, Inc.). Intensities along the nuclear rim were measured in NIH image (http://rsb.info.nih.gov/nih-image/). Measured values were analyzed in Excel (Microsoft). In most cases, fluorescence intensities were normalized from 0 to 1 corresponding to initial and final values.

Concentration and flux calculations in oocytes

The initial cytoplasmic dextran concentrations in oocytes were determined by dividing the amount of injected dextran by the measured cytoplasmic volume (taking into account that 50% of the cytoplasmic volume is occupied by yolk platelets, compare Fig. 1 D). Nuclear concentrations were then determined at later time points from the ratio of the actual nuclear and initial cytoplasmic background-subtracted mean fluorescence intensities. Total nuclear amounts were calculated by multiplying the actual concentration with the measured nuclear volume. Fluxes (number of molecules entering the nucleus per second) were then calculated from the change in dextran amount divided by the elapsed time in successive frames. We first used the real
Table SI

| Molecular weight | Concentration in oocytes | Fluorescent label | Purchased from |
|------------------|-------------------------|-------------------|---------------|
| kD               | µM                      |                   |               |
| 500              | 0.28                    | A 488             | Molecular Probes |
| 160              | 0.86                    | TRITC             | Sigma-Aldrich |
| 90               | 0.18                    | Cy 5              | Molecular Probes |
| 70               | 1.64                    | TMR               | Molecular Probes |
| 25               | 2.90                    | A 488             | Molecular Probes |
| 25               | 1.22                    | Cy 5              | Molecular Probes |
| 10               | 29.41                   | A 488             | Molecular Probes |

*DInitial cytoplasmic concentration corrected for the volume occupied by yolk platelets.

Experimental dextran concentrations (shown in Table SI). To make the fluxes comparable for different dextrans that had different initial concentrations, we show values normalized to 2 µM initial cytoplasmic concentration for all dextrans (see Fig. 3C). This normalization is valid, because the entry kinetics are not dependent on the concentration in the ~0.2–2 µM range (see Fig. S6; unpublished data). Independent experiments and calculations were averaged by normalizing the time points to 15-s intervals by linear interpolation. Identical calculations were done for MBP (see Fig. S2).

Local fluxes were calculated similarly during phase II; however, it has to be taken into account that here the entry is not homogenous but occurring at a well-defined region of the NE. Thus, we measured the permeabilized area and divided the number of dextran molecules, which entered the nucleus by this value (in case of 2D datasets we measured the radius of the permeabilized region and calculated the area of the corresponding spherical cap). Also, since the second phase is much faster the time needed for dextran molecules, which entered the nucleus by this value (in case of 2D datasets we measured the radius of the permeabilized area and divided the number of molecules that enter the nucleus per second at the end of the phase I (see above and Fig. 3C) correspond—by taking the surface of an average nucleus 2 × 104 µm²—to a flux of 50 molecules µm⁻² s⁻¹.

Pore radii that would be able to support the measured fluxes were calculated using Fick’s first law approximating dextrans as rigid spheres with radius RG passing through cylindrical pores and assuming pure diffusion as driving force. Because dextrans are comparable in size to the pore, we introduced a correction term which takes into account the fact that the mass center of a globular molecule with a radius RG cannot be closer to the pore wall than its radius, therefore the effective pore radius is reduced to R - RG (Deen, 1987; Ribbeck and Görlich, 2001). Therefore:

\[ J = \frac{D \pi R^2 \Delta c}{L} \cdot (1 - \frac{R_G}{R})^2, \]

where \( J \) is the flux (1 molecule pore⁻¹ s⁻¹), \( D \) is the diffusion coefficient in the cytoplasm, which was estimated as 0.25 × D_water (Seksek et al., 1997), \( R \) is the radius of the pore, \( \Delta c \) is 2 µM (1,200 molecules µm⁻³), \( L \) is the length of the pore (40 nm for NPC; Ribbeck and Görlich, 2001), \( R_G \) is gyration radius.

Calculated pore diameters (= 2 × R) values are shown in Table I.

GFP constructs

pEGFP2-Nup153-GEM was constructed by ligating an Agel-SmaI fragment encoding for EGFP2-Nup153 obtained from pEGFP2-Nup153 (Daigle et al., 2001) into the XmaI-SmaI sites of pGEMHE (Liman et al., 1992). pPOM121-EGFP3-GEM was made by ligating a BglII-XbaI fragment from pPOM121-EGFP3 (Daigle et al., 2001) into the BamHI-XbaI sites of pGEMHE. To generate pEGFP-LaminB1-GEM, an Agel-BamHI fragment from pEGFP-LaminB1 (Ellenberg et al., 1997) was inserted into the XmaI-BamHI sites of pGEMHE. Nup214/CAN-GFP was cloned as follows: pBS-Nup214 (von Lindern et al., 1992) was digested by SacI-Bsp120I and ligated into those sites in pEGFP-C2 (CLONTECH Laboratories, Inc.). To produce pNup214-GFP-GEM, a fragment encoding GFP-Nup214 was cut with Agel-MfeI and ligated into Xmal-EcoRI sites of pGEMHE. For Nup98, pRN3P-hsNup98, a gift from Elisa Izaurralde, was digested with EcoRI-Bsp120I and cloned into pEGFP-C2, then the Agel-XbaI fragment was inserted into XmaI-XbaI sites of pGEMHE. For in vitro transcription of mRNAs, DNA templates were linearized using Sphi for Nup153, Nup214, POM121, and Nhel for Lamin B1, Nup98, respectively.

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