Title:

Capturing Metabolism-Dependent Solvent Dynamics in the Lumen of a Trafficking Lysosome

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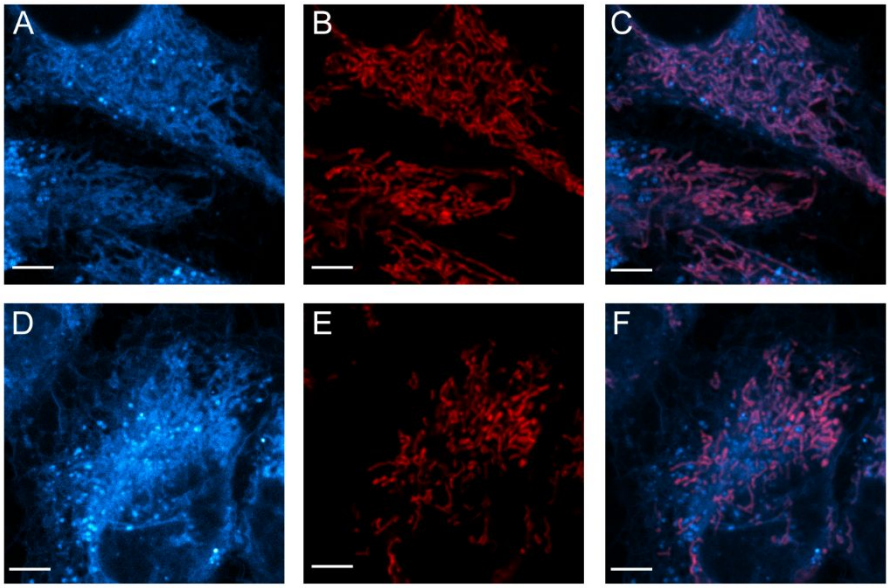

Figure S1. ACDAN localization within mitochondria in HeLa cells. Confocal microscopy images of HeLa cells labelled with ACDAN (**A** and **D**) and MitoTracker (**B** and **E**), and the corresponding overlay of the two signals (**C** and **F**). Punctuate-like signal from lysosomes has been saturated to better show the signal contribution from mitochondria. Scale bars: 5 μm.

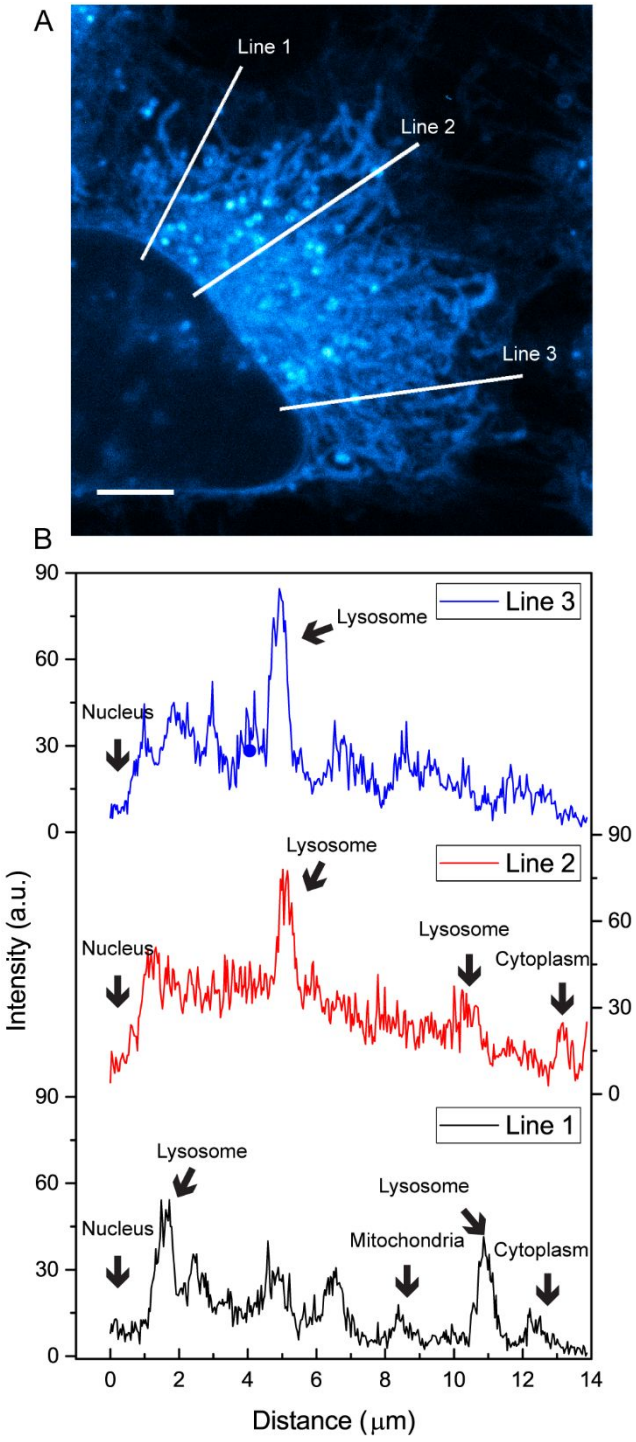

Figure S2. ACDAN distribution in HeLa cells. (**A**) Confocal microscopy images of a cell labelled with ACDAN. Scale bar: 2 μm. (**B**) Normalized intensity profiles along the white lines in (A) showing the different brightness of ACDAN within different compartments. Typically, lysosomes>mitochondria>cytoplasm>nucleus.

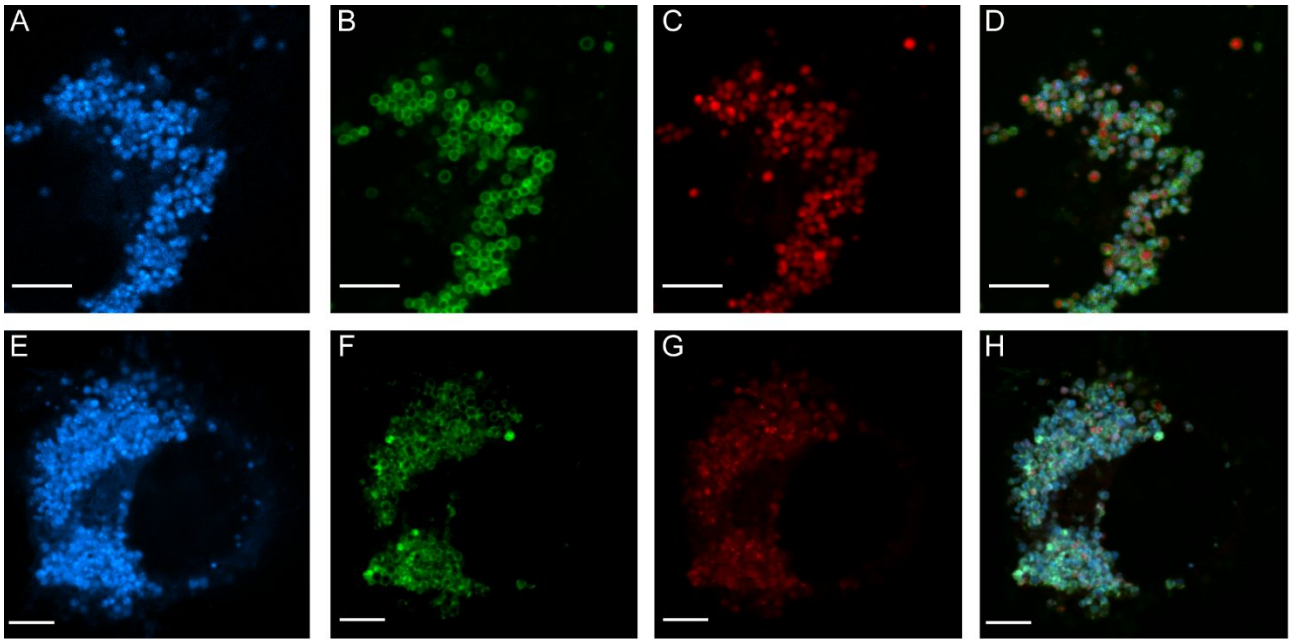

Figure S3. ACDAN colocalization with Lysotracker in the lumen of lysosomes in living HeLa cells. Confocal microscopy images of HeLa cells labelled with ACDAN (**A** and **E**), CD63-GFP (**B** and **F**) and LysoTracker (**C** and **G**), and the corresponding overlay of the three signals (**D** and **H**). Scale bars: 5 μm. Please note that ACDAN better colocalizes with Lysotracker signal arising from the lysosome lumen as opposed to CD63-GFP that, instead, is clearly labeling the lysosome membrane.

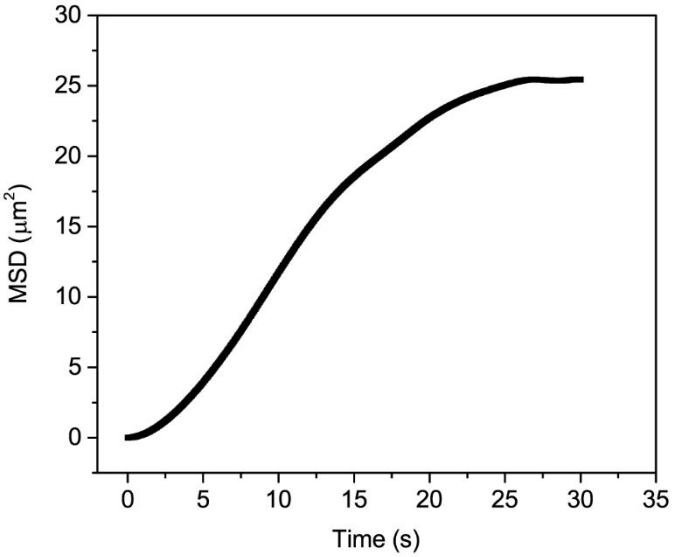

Figure S4. Average MSD curve recovered from feedback-based 3D tracking. This exemplary MSD, obtained by tracking a single lysosome, shows the contribution of directed motion at a short spatiotemporal scale (<5 s), and overall organelle confinement (or sub-diffusive behavior) at a larger spatiotemporal scale (>15 s).

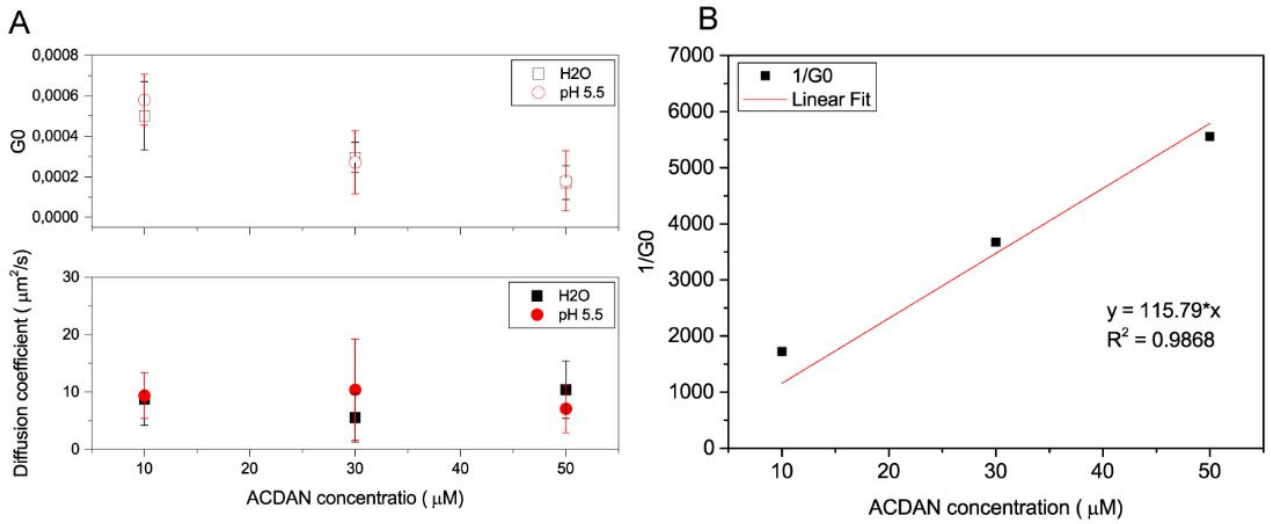

Figure S5. Calibration curves for concentration and viscosity analysis. (**A**) As expected, the G_0 value decreases as ACDAN concentration increases in aqueous solution of 66% sucrose both at neutral (black) and acidic (red) pH (top panel). Diffusion coefficients instead remain constant (lower panel). (**B**) Calibration of $1/G_0$ in 66% sucrose solution at pH=5.5.

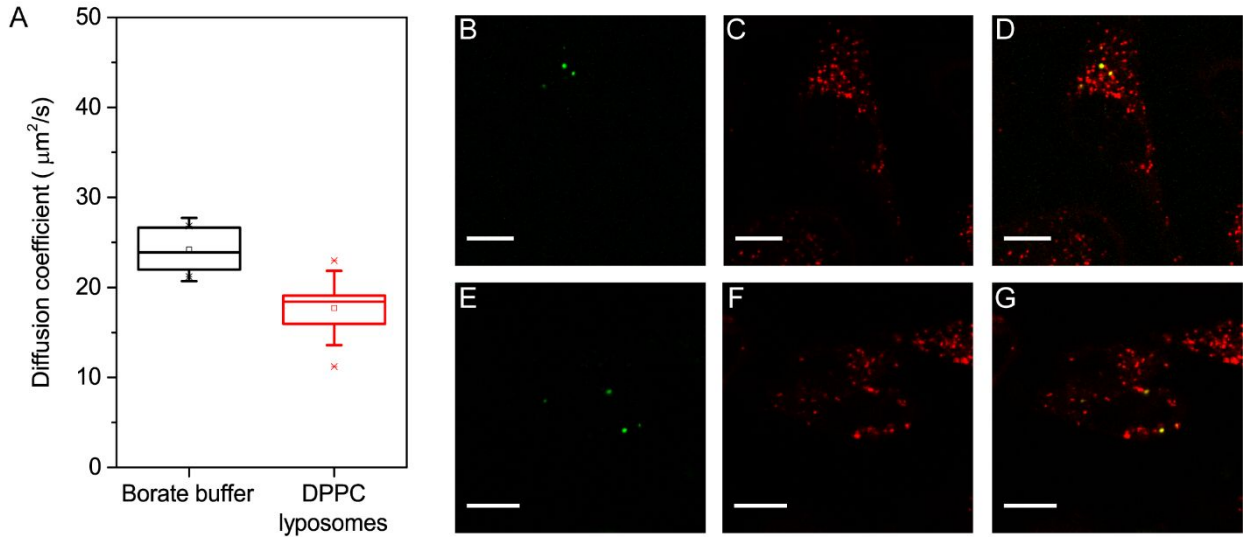

Figure S6. QDs in calibration solutions and in HeLa cells. (**A**) Diffusion coefficients of QDs in Borate buffer and in DPPC liposomes suspended in Borate buffer. These values were used for the calibration of QDs hydrodynamic radius, as described in the Methods section. (**B-G**) Confocal microscopy images of QDs (**B** and **E**) within lysosomes labelled with Lysotracker (**C** and **F**), and the corresponding overlay of the two signals (**D** and **G**). Scale bar: 10 μm.

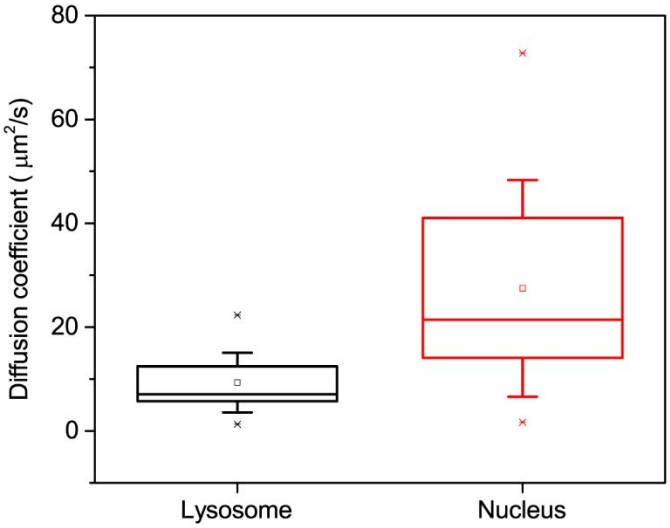

Figure S7. Diffusion coefficient of ACDAN within lysosomes (black) and nuclei (red) of HeLa cells. Upper and lower edges of the boxes represent the 25 and 75 percentile of the distributions, respectively; the middle line indicates the median value; the squares inside the boxes are the mean values; whiskers show standard deviations.

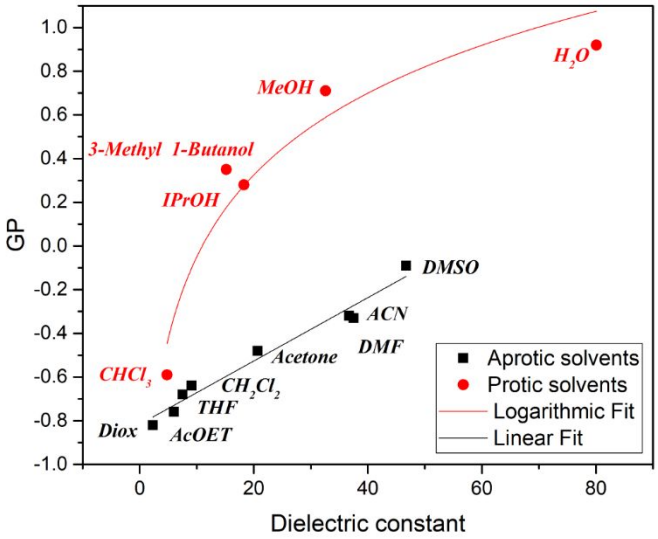

Figure S8. Dependence of ACDAN GP from the dielectric constant of the solvent. Plot of ACDAN GP as a function of the solvent dielectric constant, collected by using both protic (red dots) and aprotic (black squares) solvents.

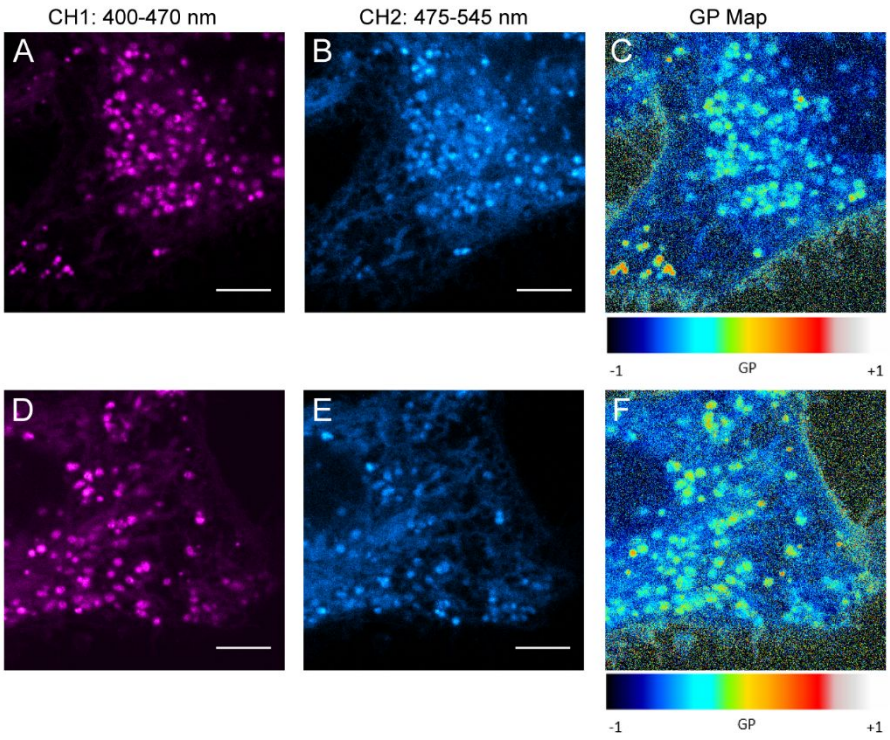

Figure S9. Imaging of ACDAN GP inside HeLa cells. Confocal microscopy images of HeLa cells labelled with ACDAN. Channel 1 (**A** and **D**, collection range: 400-470 nm) and Channel 2 (**B** and **E**, collection range: 475-545 nm) are used to quantitatively produce the GP map (**C** and **F**), as described in the Methods section. GP values are color-coded according to the LUT reported on the bottom.

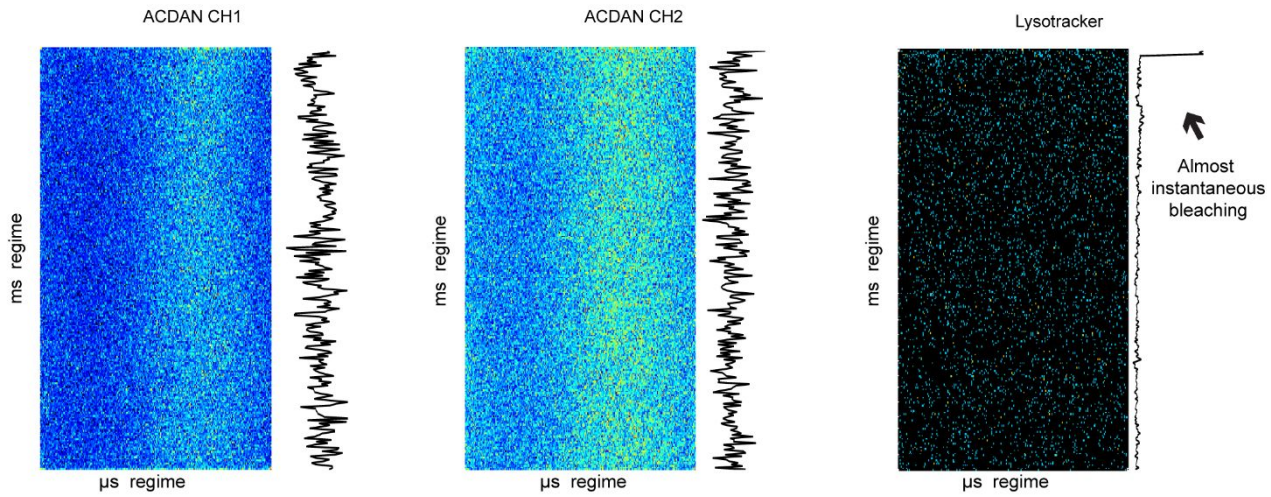

Figure S10. Comparison of ACDAN and Lysotracker carpets acquired by 3D feedback-based tracking. The carpet on the left corresponds to the signal of the first acquisition channel (CH1, collection range: 400-470 nm); the carpet in the middle represents the signal of the second acquisition channel (CH2, collection range: 475-545 nm); the carpet on the right corresponds to Lysotracker signal. It is worth noting that Lysotracker was used to localize the lysosomes to be tracked and then ACDAN signal was used as feedback source for tracking in light of its photophysical stability. Lysotracker signal, by contrast, was photobleached after few milliseconds from the beginning of the tracking experiment, as showed by the fluorescence intensity analysis associated with the carpet. Average intensities are reported both on the horizontal and vertical axes of each carpet.

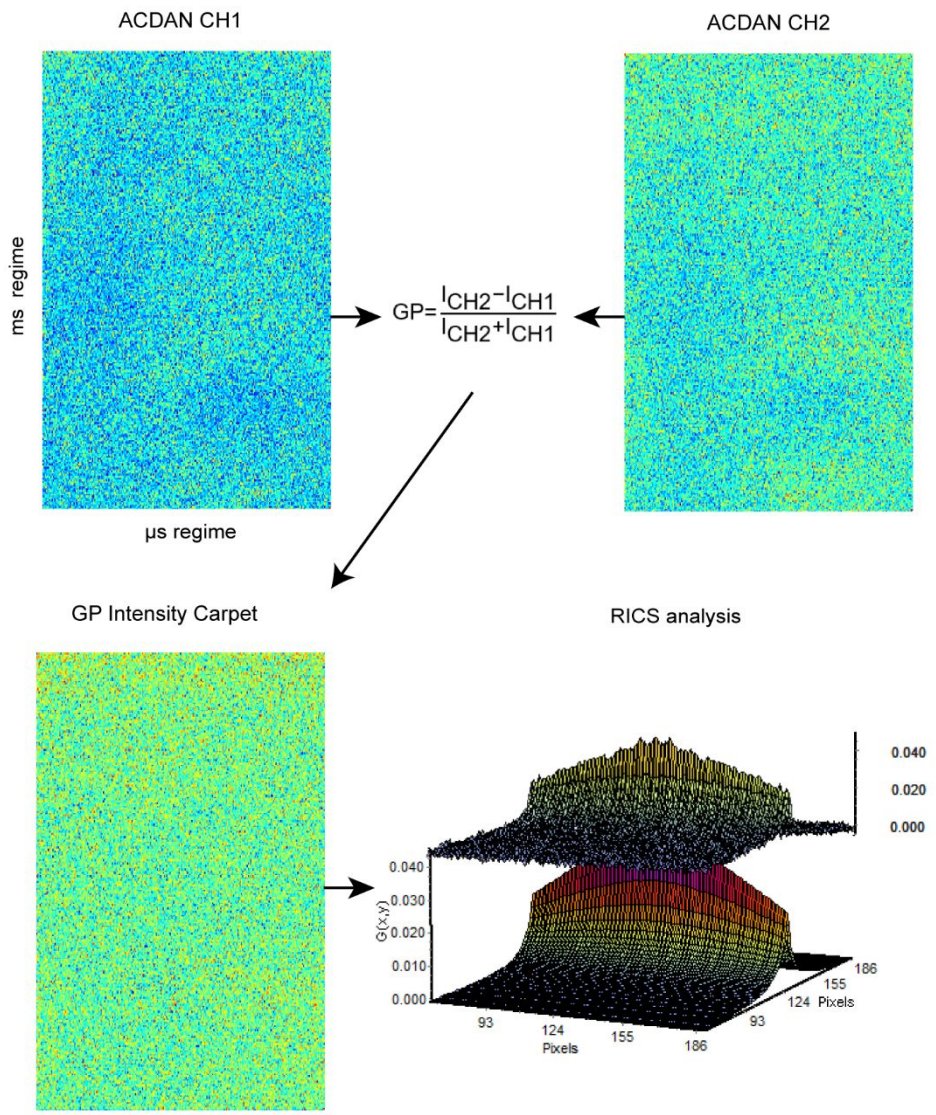

Figure S11. Schematic representation of GP analysis workflow. The two carpets of ACDAN corresponding to Channel 1 (CH1, range: 400-470 nm, top left) and Channel 2 (CH2, range: 475-545 nm, top right) are combined using Eq. 4 to obtain the GP carpet (bottom left). This carpet is then analyzed using RICS (bottom right).

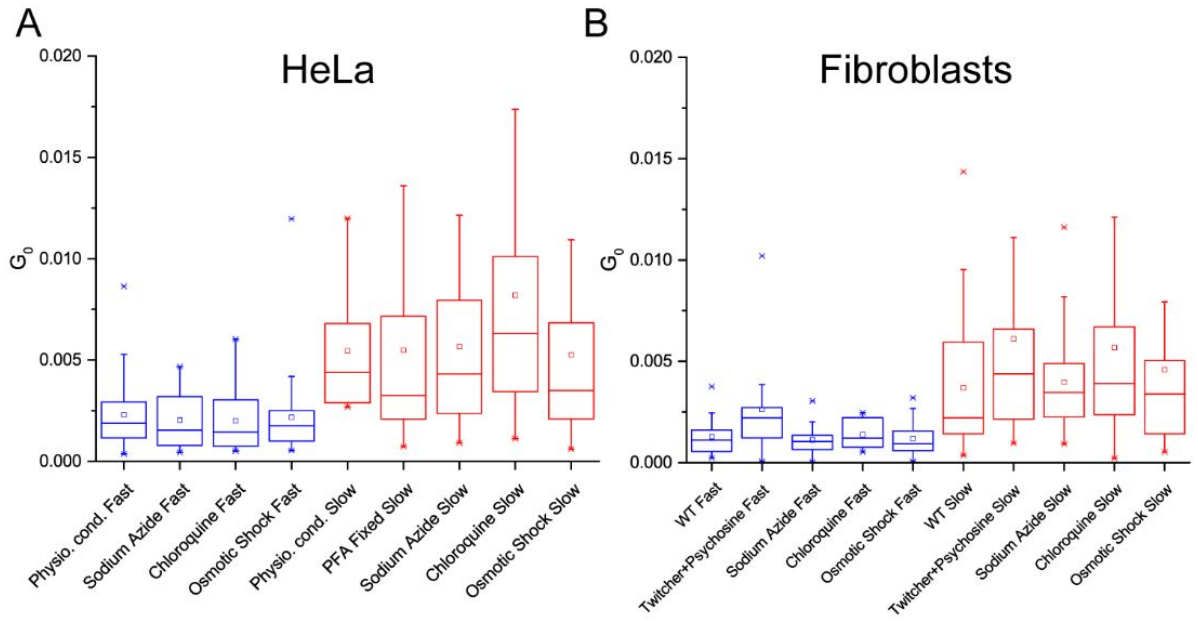

Figure S12. G_0 analysis for all the experimental conditions tested. Plot of the G_0 values of ACDAN measured inside lysosomes of HeLa cells (**A**) and fibroblasts (**B**). G_0 values of the fast population are reported in blue while those of the slow population are shown in red, for all the experimental conditions tested. Upper and lower edges of the boxes represent the 25 and 75 percentile of the distributions, respectively; the middle line indicates the median value; the square inside the box shows the mean value; whiskers are standard deviations.

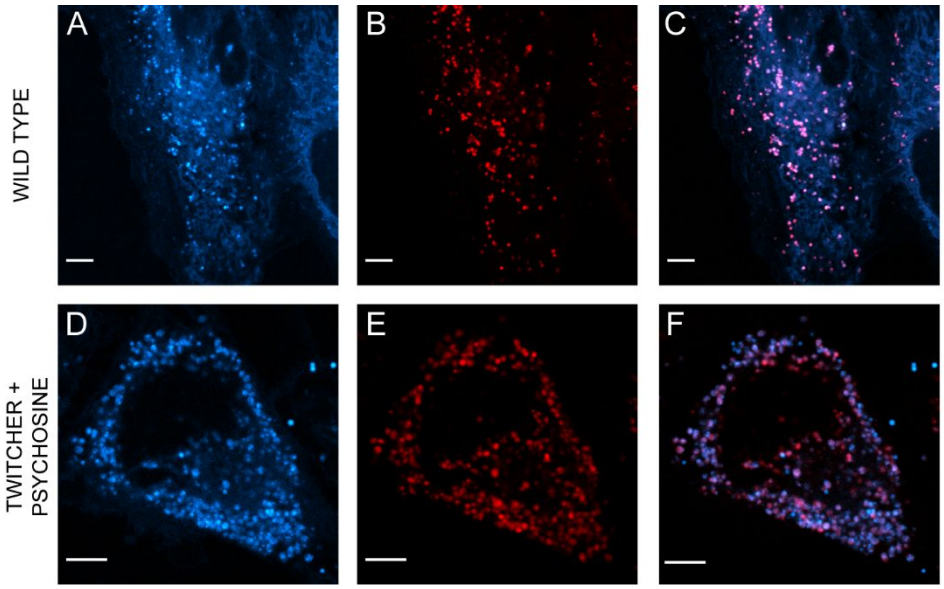

Figure S13. ACDAN localization within lysosomes in primary WT and TWI fibroblasts. Confocal microscopy images of WT fibroblasts labelled with ACDAN (**A**), Lysotracker (**B**), and the corresponding overlay of the two signals (**C**). Confocal microscopy images of TWI fibroblasts (treated with Psychosine) labelled with ACDAN (**D**), Lysotracker (**E**), and the corresponding overlay of the two signals (**F**). Scale bars: 5 µm.

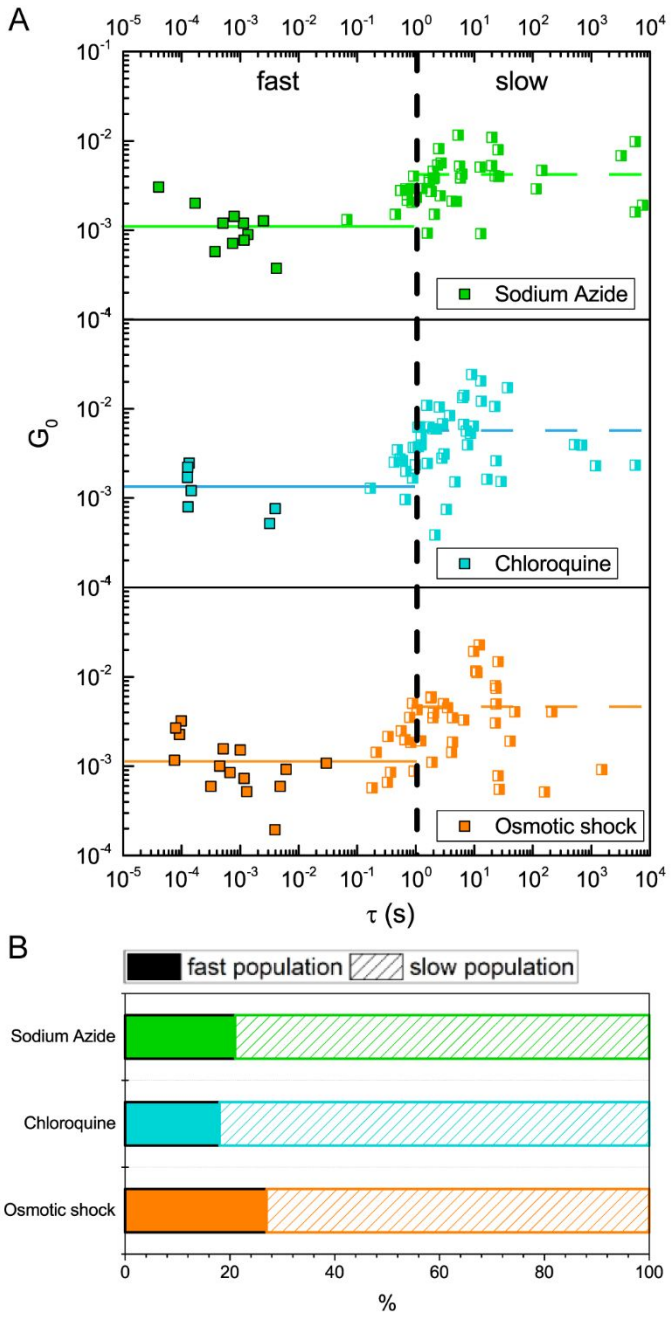

Figure S14. Circular-RICS analysis of ACDAN GP in fibroblasts. (**A**) G_0-*vs*-τ plots showing the results of the GP analysis performed on WT fibroblasts treated with Sodium Azide (green squares), Chloroquine (cyan squares) and osmotic shock (orange squares). In all plots, the dashed black line shows the limit threshold (Mean - SD) identified by the experiment on fixed HeLa cells, as explained in Fig. 3G. (**B**) Graph showing the fraction of experimental points classified as 'fast' fluctuations (filled portion of the histogram) and 'slow' (dashed portion) for all the conditions analyzed. Refer to Table 2 for a complete summary of the results obtained using WT and TWI (+Psychosine) fibroblasts.

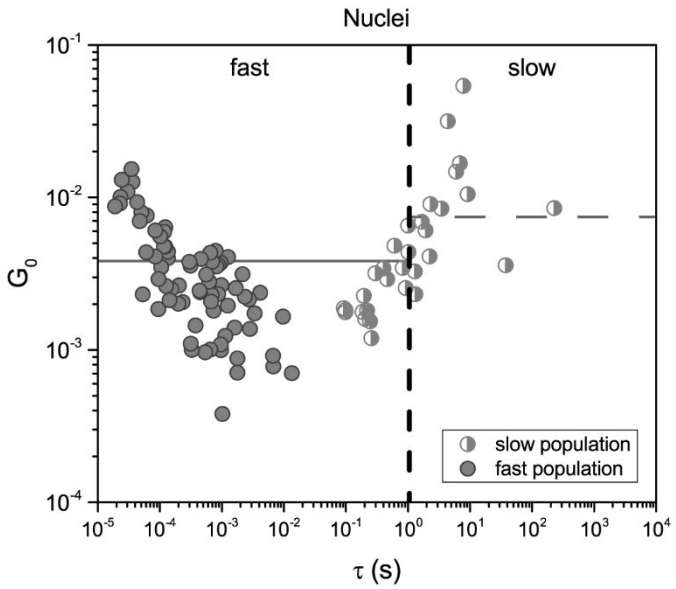

Figure S15. GP fluctuations of ACDAN in nuclei of HeLa cells. G_0-*vs*-τ plot showing the results of the GP analysis performed in the nuclei of HeLa cells under physiological conditions. Black dashed line corresponds to the Mean – SD from fixed cells, as explained in Fig. 3G.

Table S1. **Dielectric constant of solvents employed.** The Table shows the name of the solvents employed (above) and the corresponding relative dielectric constant (below).

Solvent	Diox	CHCl3	AcOET	THF	CH2Cl2	3-Methyl 1-Butanol	IPrOH	Acetone	MeOH	DMF	ACN	DMSO	H2O
Dielectric constant	0.28	4.8	6	7.52	9.1	15.2	18.3	20.7	32.6	36.7	37.5	46.7	80.1