Supporting Information

Spatiotemporal analysis of K-Ras plasma membrane interactions reveals multiple high order homo-oligomeric complexes

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Running Title: K-Ras Oligomerization

Fluorescence Correlation Spectroscopy (FCS) data analysis Fluorescent molecules freely diffusing in solution or cytoplasm were fitted using the single component 3D Gaussian diffusion model (Eq.1):

$$G(\tau) = \frac{1}{N} \cdot \frac{1}{(1+\frac{\tau}{\tau_D})} \cdot \frac{1}{(1+S^2\frac{\tau}{\tau_D})^{1/2}} \quad (1)$$

where G(τ) is autocorrelation function, τ is the observation time, τ_D is the translational diffusion time and N the average number of diffusing molecules in the confocal volume. S is structural parameter that defines the aspect ratio of the elliptical confocal volume. Free fluorescein dye in solution was used to determine the value of S (typically around 5), which was used for analysis of all the protein data. We excluded the triplet contribution in our analyses and hence the data acquired in the first 10 μs was not considered during data fitting. τ_D is related to the diffusion coefficient *D* of a molecule as (Eq. 2):

$$\tau_D = \frac{\omega^2}{4D} \quad (2)$$

As in the case of S, the observation volume ω was obtained from FCS measurements of free fluorescein dye whose diffusion coefficient is well established (D = 4.25 × 10^{-10} m^2 s^{-1}). Free fluorescein in solution and cytoplasmic cyt-GFP could be fit using the single component 3D Gaussian diffusion model of Eq. 1 (data not shown).

For our membrane–bound proteins mem-EGFP and EGFP-K-Ras that diffuse on the 2D membrane plane, we used the 2D diffusion model:

$$G(\tau) = \frac{1}{N} \cdot \left(\sum_i \frac{a_i}{1+\frac{\tau}{\tau_{Di}}} \right) \quad (3)$$

where, τ_{Di} is the diffusion time of the i^{th} species in the membrane and a_i is its fraction, which satisfies the normalization condition

$$\sum_i a_i = 1 \quad (4)$$

As shown in Figure S1, G(τ) for mem-EGFP and WT K-Ras could not be fitted with the single-component diffusion model but rather by a bi-component model, suggesting the existence of at least two diffusive species. The fast diffusing species have diffusion times of 200 - 400 μs (and correspond to GFP photophysics)[1], whereas the slower species have diffusion times that correspond to previously reported membrane protein dynamics [2,3]. However, unlike mem-EGFP, we found that a significant fraction of the FCS data we obtained for K-Ras could not be fit well even to a bi-component model and required by 3-component model (Figure S2). Here, the

fastest third component could not be explained by our experimental setup but the other two components were found to similar to what we obtained from the bi-component model.

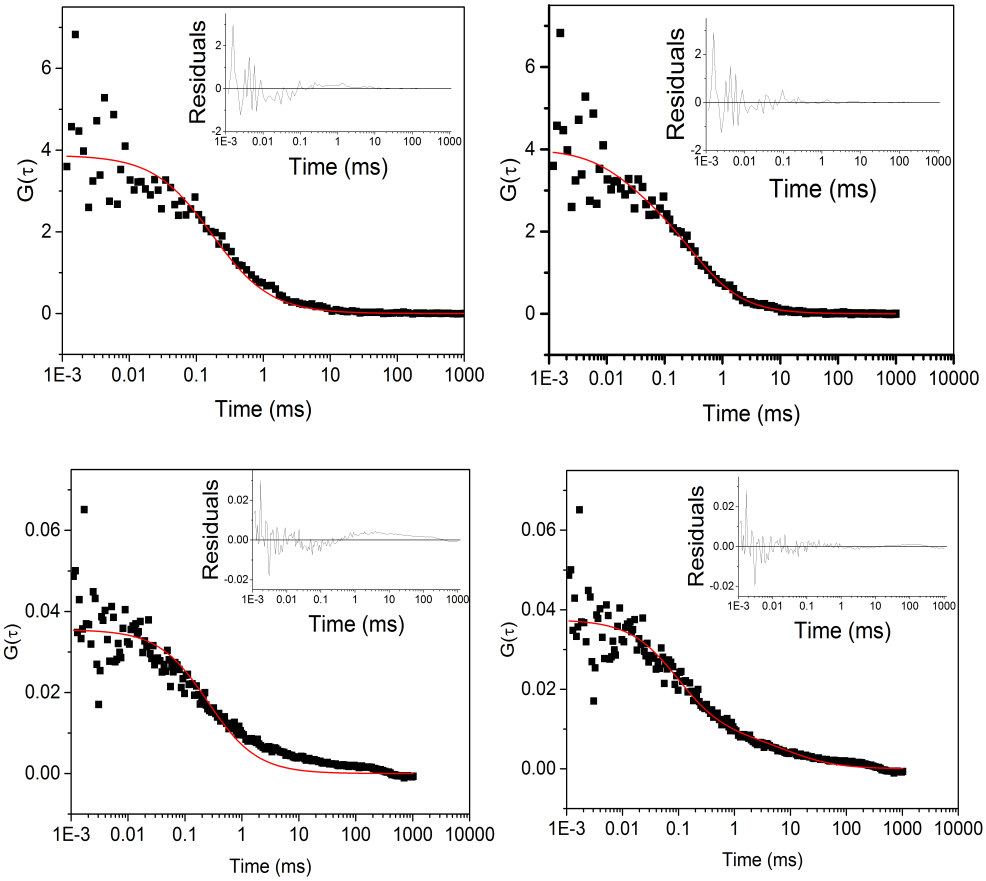

Figure S1: Comparison of single (left) and bi-component (right) fittings of mem-EGFP (top) and WT K-Ras (bottom). The 2D model provided a better fit especially for K-Ras as evident from the randomness of the residual distributions (insets).

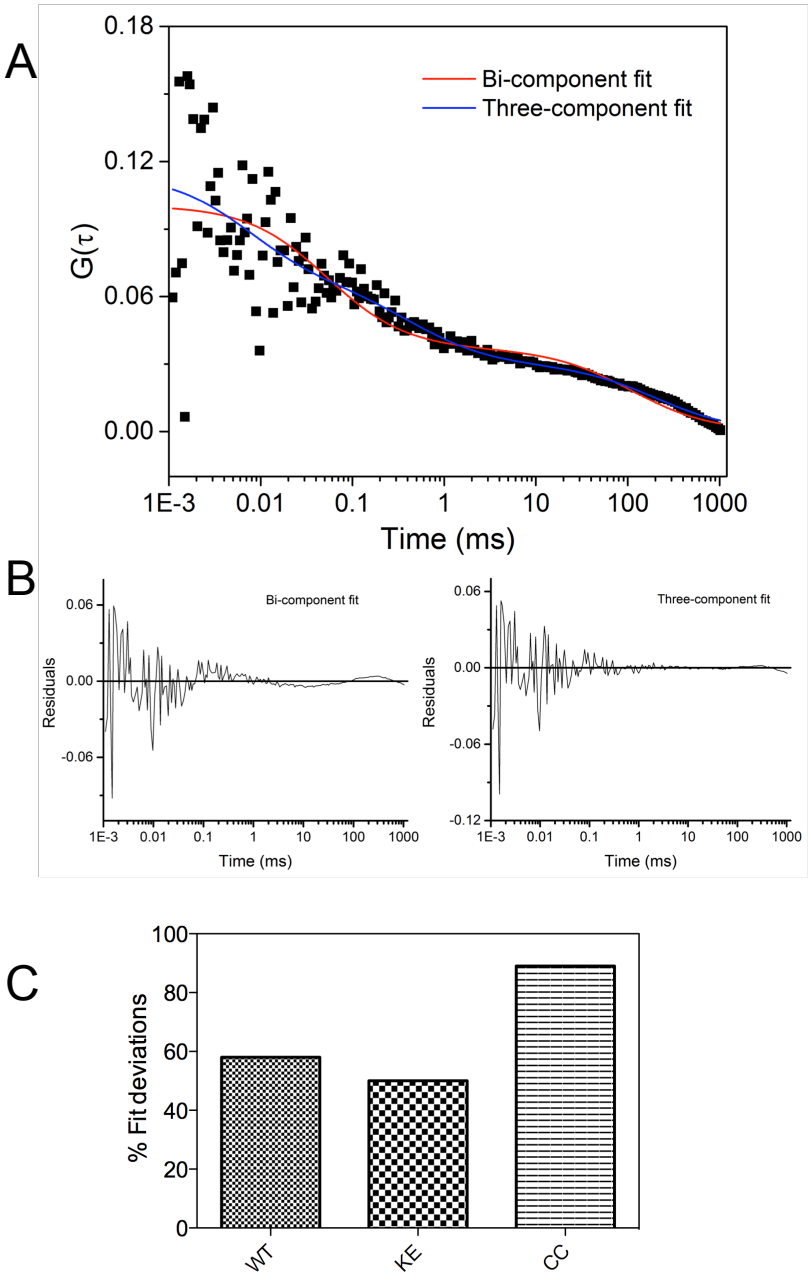

Figure S2: (**A**) An example of an atypical G(τ) profile that could be fit well by a 3-component but not by a 2-component diffusion model. (**B**) Residuals of the bi-component (left) and 3-component fits (right). (**C**) The percentage of cells analyzed in this work that yielded atypical G(τ) profiles for WT, KE and CC K-Ras.

Raster image correlation spectroscopy (RICS) and number & brightness (N&B) analysis

The RICS data was processed as described in ref. [4] based on the 2D spatial correlation of the fluorescent intensity in each frame (Eq. 5),

$$G_{RICS}(\xi,\psi) = \frac{<I(x,y)I(x+\xi,y+\psi)>}{<I(x,y)>^2} \quad (5)$$

where (ξ, ψ) are the spatial lag time in the horizontal and vertical (x,y) dimensions, respectively. $G_{RICS}(\xi,\psi)$ can be expressed in terms of $S(\xi,\psi)$, which depends on scanning parameters such as the excitation volume and the pixel size, and $G(\xi,\psi)$, which contains information about the diffusion of the fluorescent molecule, as [5]:

$$G_{RICS}(\xi,\psi) = S(\xi,\psi)G(\xi,\psi) \quad (6)$$

where

$$S(\xi,\psi) = exp\left(-\frac{\left(\frac{\xi\delta r}{\omega_0}\right)^2+\left(\frac{\psi\delta r}{\omega_0}\right)^2}{1+\frac{4D(\tau_p\xi+\tau_l\psi)}{\omega_0^2}}\right) \quad (7)$$

and

$$G(\xi,\psi) = \frac{\gamma}{N}\left(1+\frac{4D(\tau_p\xi+\tau_l\psi)}{\omega_0^2}\right)^{-1}\left(1+\frac{4D(\tau_p\xi+\tau_l\psi)}{\omega_0^2}\right)^{-1/2} \quad (8)$$

γ is the illumination profile factor assumed to be 0.35 for a 3D Gaussian using one-photon excitation, and N denotes the apparent number of fluorescent species in the excitation volume. While calculating a diffusion coefficient using RICS, slow background motions associated with the movement of organelles and/or the cell are filtered out by applying a moving average of 10.

N&B analysis allows for the quantification of the fraction of oligomeric species from the brightness map [6]. Since N&B analysis of an image allows for distinguishing pixels containing very dim molecules from those with few bright molecules, it can be used to quantify the degree of oligomerization of the fluorescent species from the average and variance of the intensity distribution. The number of molecules (N) and the brightness (B) are related to the average (<k>) and variance (σ^2) of the intensity distribution in any given pixel via Eqs. 9 and 10, where the average is calculated for the same pixel in the image stacks.

$$N = \frac{<k>^2}{\sigma^2} = \frac{\varepsilon}{\varepsilon+1}n, \quad (9)$$

$$B = \frac{\sigma^2}{<k>} = \varepsilon + 1 \quad (10)$$

where n is the number of particles in the excitation volume and ε is the molecular brightness. The variance arises from particle fluctuations or from detector shot noise. The immobile

molecules do not have particle fluctuation and thus their apparent brightness B equals 1 and is independent of the laser power, whereas that of the mobile fraction is laser power-dependent.

Parameters such as detector *offset*, the factor *S* that converts photon counts to digital levels, and the readout variance σ_0^2 are needed in N&B analysis as expressed in Eqs 11 and 12:

$$N = \frac{(<k>-offset)^2}{\sigma^2-\sigma_0^2} = \frac{\varepsilon}{\varepsilon+1}n, \quad (11)$$

$$B = \frac{\sigma^2-\sigma_0^2}{<k>-offset} = S(\varepsilon+1) \quad (12)$$

In our case, S was set to 1 while the offset and σ_0^2 were both set to 0 [7]. According to Digman et al. [8], B=1 indicates that an immobile fraction is present in the pixel. The contribution from any immobile species to the molecular brightness is corrected using Eq. 13:

$$\varepsilon_m = \frac{\varepsilon}{1+R} \quad (13)$$

where ε_m is the molecular brightness of the mobile species and *R* is the intensity ratio between the immobile and mobile fractions per pixel.

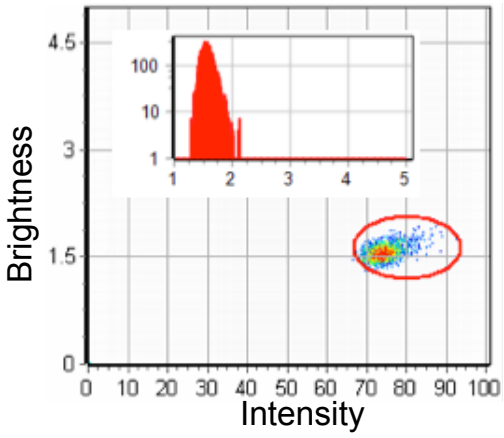

Figure S3: Brightness distribution (B-map, main figure) and histogram (inset) for mem-EGFP of the region in the red circle. The single sharp peak suggests the existence of only one fluorescent species.

Table S1: Diffusion coefficients (D) of WT and mutant K-Ras ($\mu m^2/s$).

Protein	FRAP[a]		FCS[b]	RICS
mem-EGFP	-	-	-	7.22 ± 3.28
EGFP-LactC2-PS	-	-		4.70 ± 2.29
WT	0.11-0.36 (46)	0.04-0.07 (54)	0.57 ± 0.67	2.19 ± 0.66
KE	0.31-0.45 (62)	0.03-0.09 (38)	0.91 ± 0.29	5.08 ± 2.16
CC	0.27-0.42 (61)	0.03-0.09 (39)	0.21 ± 0.18	1.35 ± 0.71

[a] 55, 80 and 88% of the FRAP measurements could be fit well to a bi-component diffusion model yielding fast (D_1, first column) and slow (D_2, second column) rates of diffusion; numbers in brackets represent the relative contribution of the two to the overall dynamics. [b] For comparison, we obtained D = 72-78 $\mu m^2/s$ for purified GFP in solution, and $D \approx 27$ $\mu m^2/s$ for cytosolic EGFP (cyt-GFP). D ± S. D. is given for FCS and RICS and ranges for FRAP.

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