

SUPPLEMENTARY INFORMATION

Probing biomolecular condensate microenvironments with multimodal fluorescence anisotropy and refractive index-driven lifetime modulation

Miguel Ángel Sacristán,^[a,b,c] Elena Tapia,^[a] Jeff Coleman,^[d] M. Pilar Lillo,^[e] and Iván López-Montero^{*[a,b,c]}

[^a] Departamento de Química Física, Universidad Complutense de Madrid, Av. Complutense s/n 28040 Madrid, Spain

[^b] Instituto Pluridisciplinar, Universidad Complutense de Madrid, Paseo Juan XXIII 1, 28040 Madrid, Spain

[^c] Instituto de Investigación Hospital Doce de Octubre (imas12), Av. Córdoba s/n, 28041 Madrid, Spain

[^d] Department of Cell Biology, School of Medicine, Yale University, 333 Cedar Street, New Haven, CT, USA

[^e] Instituto de Química Física Blas Cabrera, Consejo Superior de Investigaciones Científicas, C/ Serrano 117, 28006 Madrid, Spain

E-mail: ivanlopez@quim.ucm.es

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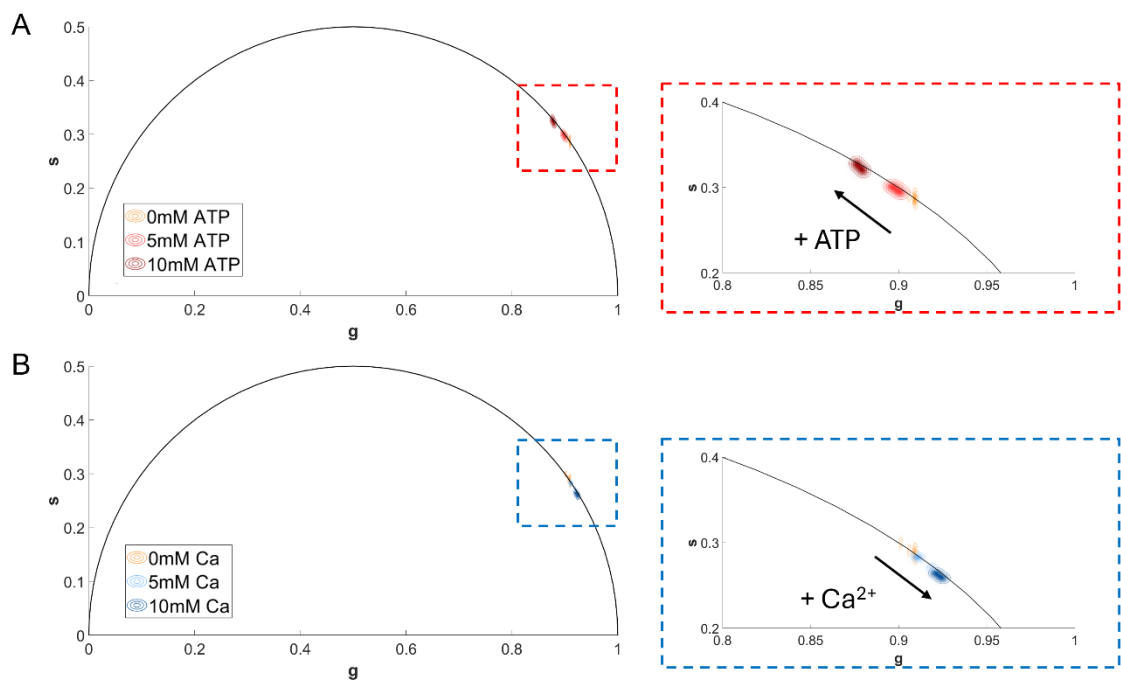


Figure S1. FLIM phasor analysis of mNG-DDX4(1–236) condensates under ATP and Ca^{2+} modulation. **A)** Phasor plot of condensates measured at increasing ATP concentrations. **B)** Phasor plot of condensates measured at increasing Ca^{2+} concentrations. The clustering and displacement of the phasor coordinates are consistent with the monoexponential fluorescence lifetime analysis presented in the main text, providing a model-independent visualization of the lifetime changes induced by ATP and Ca^{2+} .

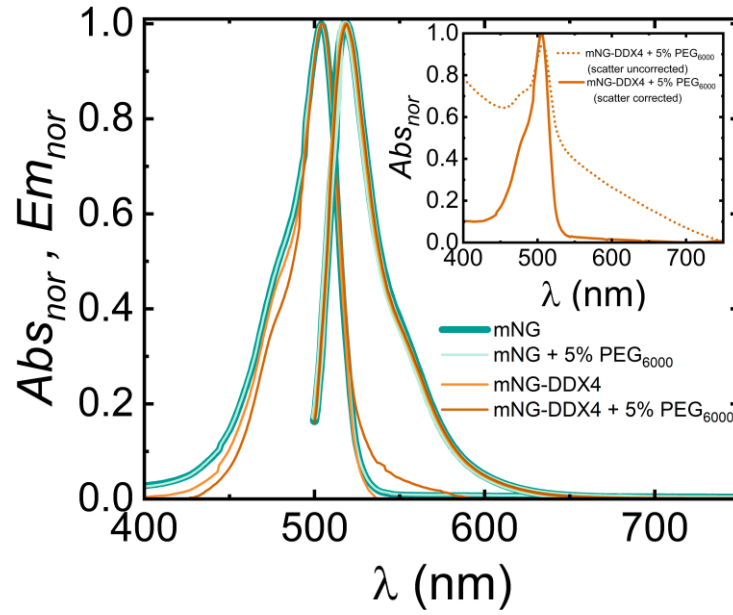


Figure S2. Absorption and emission spectra of mNeonGreen (mNG) and mNG-DDX4(1-236). Absorption and emission spectra of free mNG in aqueous solution and in the presence of 5% (w/v) PEG6000, compared with spectra of mNG-DDX4 under the same conditions. All spectra largely overlap, indicating that neither PEG nor fusion to DDX4 significantly perturbs the intrinsic photophysical properties of mNG, with excitation and emission maxima at $\lambda_{ex,max} = 500 \text{ nm}$ and $\lambda_{em,max} = 520 \text{ nm}$, respectively. **Inset:** Absorption and emission spectra of mNG-DDX4 condensates in 5% PEG6000, before and after scatter correction, demonstrating that spectral shape is preserved upon phase separation while correcting for light scattering artifacts.

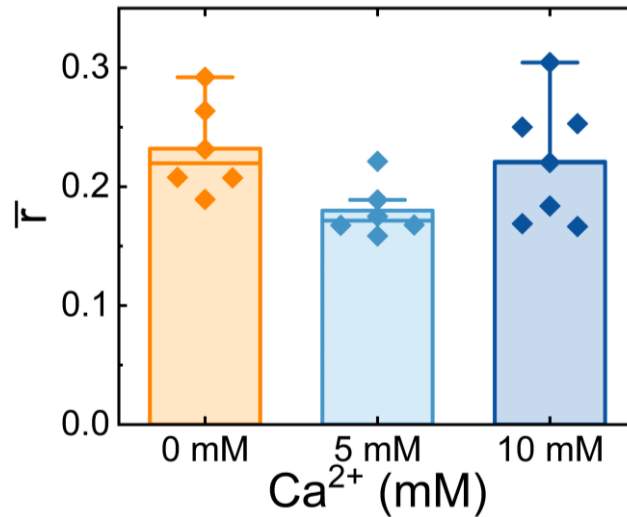


Figure S3. Effect of Ca^{2+} on fluorescence anisotropy in DDX4(1-236) condensates containing a low fraction of fluorescently labelled protein. DDX4 condensates were prepared using a mixture containing 5% mNG-DDX4(1-236) and 95% unlabelled DDX4(1-236) to reduce homoFRET while preserving the overall protein density. Steady-state fluorescence anisotropy was measured at increasing Ca^{2+} concentrations. No statistically significant changes in anisotropy were observed, indicating that the absence of anisotropy changes under Ca^{2+} conditions is not caused by saturation of the homoFRET signal. Bars represent the mean, error bars indicate standard deviation (SD), and horizontal lines indicate the median.

Models for quantitative characterization of condensate density.

A. Protein density quantification through steady state fluorescence anisotropy. Homo-FRET processes enhance fluorescence depolarization through non-radiative energy transfer between identical fluorophores. Upon excitation of a donor molecule, energy can be transferred to a nearby ground-state acceptor whose transition dipole moment is generally differently oriented in space [1]. Because the emission dipole of the acceptor is angularly uncorrelated with the initially excited donor dipole, each transfer event contributes to a partial loss of polarization memory, resulting in a reduction of the steady-state fluorescence anisotropy $\bar{r}$.

For an isotropic distribution of fluorophores interacting through long-range dipole–dipole coupling, the pairwise Förster transfer rate is given by

$$k_{\text{FRET}}(R) = \frac{1}{\tau} \left(\frac{R_0}{R} \right)^6 \quad (\text{Eq. S1})$$

where τ is the intrinsic fluorescence lifetime in the absence of energy transfer, R_0 is the Förster radius, and R is the intermolecular separation. The transfer rate therefore scales as R^{-6} . Because intermolecular distance depends on molecular density, $R \equiv R(\rho)$, the overall depolarization becomes an implicit function of fluorophore concentration.

In concentrated systems such as biomolecular condensates, each donor can interact simultaneously with multiple acceptors. In this regime, the depolarization process depends not only on pairwise distances but on the full three-dimensional spatial distribution of fluorophores. Consequently, steady-state anisotropy reflects the geometric packing density of fluorescent proteins.

To extract protein density from steady-state anisotropy, we adopt the theoretical framework proposed by Bodunov [2]. The model assumes that the dominant contribution to measured anisotropy arises from directly excited molecules, whereas emission originating from secondary excited fluorophores is completely depolarized. Under this approximation, steady-state anisotropy becomes proportional to the fraction of fluorescence arising from directly excited donors. This leads to the relation

$$\frac{\bar{r}}{\bar{r}^*} = \frac{\phi}{\phi^*} \quad (\text{Eq. S2})$$

where $\bar{r}$ is the measured steady-state anisotropy in the presence of homo-FRET, $\bar{r}^*$ is the anisotropy that would be observed in the absence of energy transfer (but still accounting for viscosity and rotational diffusion), ϕ is the apparent quantum yield of directly excited molecules, and ϕ^* is the intrinsic quantum yield of the fluorophore. For fluorescent proteins, the quantum yield can be considered independent of refractive index [3], allowing the ratio of quantum yields to be expressed as the ratio of time-integrated fluorescence decays:

$$\frac{\phi}{\phi^*} = \frac{\int {}^1I(t)dt}{\int I(t)dt} \quad (\text{Eq. S3})$$

where ${}^1I(t)$ is the fluorescence decay of directly excited molecules and $I(t)$ is the fluorescence decay in the absence of energy transfer. For a monoexponential decay,

$\int I(t) dt = I_0 \tau$, whereas in the presence of energy transfer the decay of directly excited fluorophores deviates from monoexponential behavior and can be written as

$$^1I(t) = I_0 e^{-t/\tau} G(t) \quad (\text{Eq. S4})$$

where $G(t)$ represents the survival probability of an excited donor at time t . For an isotropic three-dimensional distribution of acceptors, Huber, Hamilton and Barnett [4] derived the survival function

$$G(t) = \exp\left(-\frac{c}{2} \int_V (1 - e^{-2t k_{FRET}(r)}) dV\right) \quad (\text{Eq. S5})$$

Substituting the Förster rate expression and integrating over space yields a closed-form relationship between anisotropy and molecular density. The final expression can be written as

$$\frac{\bar{r}}{\bar{r}^*} = \frac{\tau_{cond}}{\tau} \left(1 - \sqrt{\pi} \gamma e^{\gamma^2} \text{erfc}(\gamma)\right) \quad (\text{Eq. S6})$$

where τ_{cond} is the measured lifetime within condensates, erfc is the complementary error function and

$$\gamma = \frac{\sqrt{2}}{3} \pi^{3/2} R_0^3 \rho_m \quad (\text{Eq. S7})$$

with ρ_m the molar protein density. This expression directly links steady-state anisotropy to fluorophore concentration.

The reference anisotropy $\bar{r}^*$ must account for rotational diffusion in the absence of energy transfer. Using the Perrin equation for monoexponential decay, it can be written as

$$\bar{r}^* = r_0 \frac{\varphi}{\varphi + \tau} \quad (\text{Eq. S8})$$

where r_0 is the fundamental anisotropy and φ is the rotational correlation time extracted from time-resolved anisotropy analysis.

For error analysis, implicit function theorem is employed. Using $R = \frac{\bar{r} \tau}{\bar{r}^* \tau_{cond}}$, the variance of γ is:

$$\sigma_\gamma^2 = \frac{\sigma_R^2}{\left(-2\gamma + (1+2\gamma^2)e^{\gamma^2}\sqrt{\pi}\text{erfc}(\gamma)\right)^2} \quad (\text{Eq. S9})$$

B. Protein density quantification through fluorescence lifetime. In dilute protein solutions, the refractive index varies approximately linearly with concentration, such that dn/dc can be treated as constant [5,6]. However, biomolecular condensates reach protein concentrations in the millimolar range, where this linear approximation may no longer be valid. In such dense dielectric phases, the refractive index must instead be related to molecular polarizability through the Lorentz–Lorenz formalism [7].

The Lorentz–Lorenz equation connects refractive index to molecular polarizability as

$$\frac{n^2-1}{n^2+2} = \frac{\rho N_A \alpha}{3\epsilon_0} = \frac{N_A \alpha}{3\epsilon_0 v} \quad (\text{Eq. S10})$$

In terms of molar refractivity, R_M , this expression becomes

$$R_M = \frac{M}{\rho} \frac{n^2-1}{n^2+2} = \frac{N_A \alpha}{3\epsilon_0} \quad (\text{Eq. S11})$$

where R_M is the molar refractivity, M is the molar mass, ρ is the mass density, v is the molar volume, n is the refractive index, N_A is Avogadro's number, ϵ_0 is the vacuum permittivity, and α is the molecular polarizability.

For multicomponent systems such as biomolecular condensates, the molar refractivity is additive provided that electromagnetic coupling between components is negligible. Under this assumption, the refractive index satisfies [8].

$$\frac{n_s^2-1}{n_s^2+2} = \frac{N_A}{3\epsilon_0} \sum_i \rho_{m,i} \alpha_i \quad (\text{Eq. S12})$$

Instead of molecular polarizability α , it is convenient to introduce the polarizability volume.

$$\alpha' = \frac{\alpha}{4\pi\epsilon_0} \quad (\text{Eq. S13})$$

which is commonly reported in cubic ångström units ($\text{\AA}^3$). If the protein molar density is denoted ρ_m , the effective condensate polarizability can be written as

$$\tilde{\alpha} = \alpha_{\text{protein}} + \sum_i r_i \alpha_i \quad (\text{Eq. S14})$$

where $r_i = \rho_{m,i}/\rho_m$ represents the relative concentration of solvent component i with respect to protein. The second term defines a polarizability excess $\Delta\alpha$ arising from solvent contributions.

For a binary mixture (protein and solvent), assuming volume additivity ($V = \sum v_i n_i$)

$$\sum \rho_{m,i} v_i = 1 \quad (\text{Eq. S15})$$

which leads to

$$\rho_{m_s} = \frac{1}{v_s} - \frac{v_p}{v_s} \rho_{m_p} \quad (\text{Eq. S16})$$

Substitution into the Lorentz–Lorenz relation yields

$$n = \sqrt{\frac{8\pi N_A \left((v_s \alpha'_p - v_p \alpha'_s) \rho_{m_p} + \alpha'_s \right) + 3v_s}{3v_s - 4\pi N_A \left((v_s \alpha'_p - v_p \alpha'_s) \rho_{m_p} + \alpha'_s \right)}} \quad (\text{Eq. S17})$$

At low protein concentrations, this expression can be approximated by a Taylor series expansion. The first-order term provides the protein refractive index increment

$$\frac{dn}{dc} = \frac{18\pi N_A v_s (v_p \alpha'_s - v_s \alpha'_p)}{(4\pi N_A \alpha'_s - 3v_s)(8\pi N_A \alpha'_s + 3v_s)} \sqrt{\frac{3v_s + 8\pi N_A \alpha'_s}{3v_s - 4\pi N_A \alpha'_s}} \quad (\text{Eq. S18})$$

For dilute protein solutions, this refractive index increment provides a reliable method for estimating concentration. However, in biomolecular condensates where protein concentrations are high, convergence between the linear approximation and the full Lorentz–Lorenz expression cannot be guaranteed.

The intrinsic molecular polarizability of the protein can be approximated by summing the contributions of individual amino acids (see **Table S1**) [9]. Reported values for bovine serum albumin (BSA) range from 5800 to 7800 Å³ [10], and the present additive model yields an estimate of approximately 7300 Å³. A comparable value is obtained for mNG-DDX4(1-236). These intrinsic values are used solely as input parameters in the Lorentz–Lorenz analysis and should not be confused with the effective condensate polarizability reported in the main text.

Using $\alpha'_p = 7300 \text{ Å}^3$, $v_p = 47.1 \cdot 10^{-3} \text{ m}^3/\text{mol}$, $\alpha'_s = 1.4 \text{ Å}^3$ and $v_s = 18.0 \cdot 10^{-6} \text{ m}^3/\text{mol}$, together with the protein concentrations obtained from fluorescence anisotropy measurements, the estimated refractive index values are reported in **Table S2**.

The discrepancy in refractive index values obtained from lifetime analysis may arise from the heterogeneous composition of biomolecular condensates. The presence of highly polarizable molecules can increase the refractive index, while inter- and intramolecular interactions (e.g., π – π and cation– π interactions) may also modify the intrinsic polarizability of the protein.

Using the previously obtained values, the refractive index increment can also be estimated, yielding $\frac{dn}{dc} = 0.216 \text{ mL} \cdot \text{g}^{-1}$, slightly larger than the expected value of $0.197 \text{ mL} \cdot \text{g}^{-1}$. This difference may indicate that the intrinsic protein polarizability is somewhat lower than 7300 Å³, or that the protein molar volume is slightly larger. Nevertheless, polarizability-based analysis is expected to provide a more reliable description of biomolecular condensates than approaches relying solely on refractive index increments.

Ethylene glycol has a molar mass of $M = 62.07 \text{ g/mol}$, a mass density of $\rho = 1.11 \cdot 10^6 \text{ g/m}^3$ and refractive index of $n = 1.43$. From the Lorentz-Lorenz equation, a polarizability of $\alpha' = 5.7 \text{ Å}^3$ can be obtained. Assuming additive behavior in polymers, $\alpha'_{PEG_N} = N \times 5.7 \text{ Å}^3$, PEG600 ($N=10$) has reported a polarizability of $\alpha'_{PEG_{10}} \simeq$

$58 \text{ \AA}^3$ [11], close to our estimations of $\alpha'_{PEG_{10}} \simeq 57 \text{ \AA}^3$. Accordingly, PEG6000 can be approximated with $\alpha'_{PEG_{100}} \simeq 600 \text{ \AA}^3$. ATP also exhibits significant polarizability due to the adenine ring, whereas water and small ions have polarizabilities on the order of $1 \text{ \AA}^3$ and can be neglected to first approximation.

For a ternary mixture of protein (p), solute (c), and solvent (s), and assuming additivity of molar volumes, the refractive index can be expressed as:

$$n = \sqrt{\frac{8\pi N_A \left((v_s \alpha'_p - v_p \alpha'_s) \rho_{m_p} + (v_s \alpha'_c - v_c \alpha'_s) \rho_{m_c} + \alpha'_s \right) + 3v_s}{3v_s - 4\pi N_A \left((v_s \alpha'_p - v_p \alpha'_s) \rho_{m_p} + (v_s \alpha'_c - v_c \alpha'_s) \rho_{m_c} + \alpha'_s \right)}} \quad (\text{Eq. S19})$$

The solute concentration in the condensate can therefore be expressed as:

$$\rho_{m_c} = \frac{1}{v_c \alpha'_s - v_s \alpha'_c} \left((v_s \alpha'_p - v_p \alpha'_s) \rho_{m_p} + \alpha'_s + \frac{3}{4\pi N_A} v_s \frac{1-n^2}{n^2+2} \right) \quad (\text{Eq. S20})$$

In our system, PEG and ATP are the solutes with the most relevant polarizability. Approximating their combined contribution as that of PEG alone yields a ratio ρ_{m_c}/ρ_{m_p} of ~ 50 for ATP conditions and ~ 20 for Ca^{2+} conditions. Inclusion of ATP in the effective condensate polarizability would reduce this ratio depending on the PEG/ATP proportion in the condensate.

Thus, deviations of the effective condensate polarizability from the intrinsic protein polarizability reflect additional optical contributions arising from co-partitioning molecules such as PEG and ATP, as well as their interactions within the condensed phase. The PEG-free control experiments presented in the main text further support this interpretation, as removal of PEG decreases the effective condensate polarizability while leaving the homoFRET-derived protein density essentially unchanged.

AA	α' ($\text{\AA}^3$)	v_m (mL/mol)	AA	α' ($\text{\AA}^3$)	v_m (mL/mol)
Arg (R)	16.7	127.3	Gly (G)	5.6	43.2
His (H)	15.7	98.8	Pro (P)	10.7	81.0
Lys (K)	14.3	108.5	Ala (A)	7.4	60.4
Asp (D)	9.9	73.8	Ile (I)	12.9	107.5
Glu (E)	11.7	85.9	Leu (L)	12.9	107.5
Ser (S)	8	60.3	Met (M)	14.2	105.3
Thr (T)	9.8	76.8	Phe (F)	15	121.2
Asn (N)	11.3	78.0	Trp (W)	21.5	143.9
Gln (Q)	14.3	98.9	Tyr (Y)	16	123.1
Cys (C)	10.5	73.4	Val (V)	11.1	90.8

Table S1. Polarizability volume and molar volume of amino acids. Polarizability volume [¹²] and molar volume [¹³] values of each amino acid used to calculate protein polarizabilities and molar volume.

	10 mM ATP	5 mM ATP	1 mM ATP		1 mM Ca ²⁺	5 mM Ca ²⁺	10 mM Ca ²⁺	NO PEG
<i>n</i> FLIM	1.36	1.42	1.47	1.46	1.46	1.48	1.52	1.37
<i>n</i> estimated	1.33	1.34	1.34	1.36	1.37	1.37	1.37	1.36

Table S2. Comparison between refractive index values obtained from FLIM measurements and those estimated from the polarizability model. Refractive indices measured by fluorescence lifetime imaging microscopy (FLIM) and values estimated using the Lorentz–Lorenz–based model with protein concentrations derived from fluorescence anisotropy are shown for DDX4(1–236)-mNG condensates under different ATP and Ca²⁺ concentrations. Agreement between both approaches is markedly improved for PEG-free condensates, supporting the interpretation that PEG primarily contributes to the optical polarizability of the condensed phase rather than to protein packing.

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