Extended Data Figures for:
Single fluorogen imaging reveals spatial inhomogeneities within biomolecular condensates

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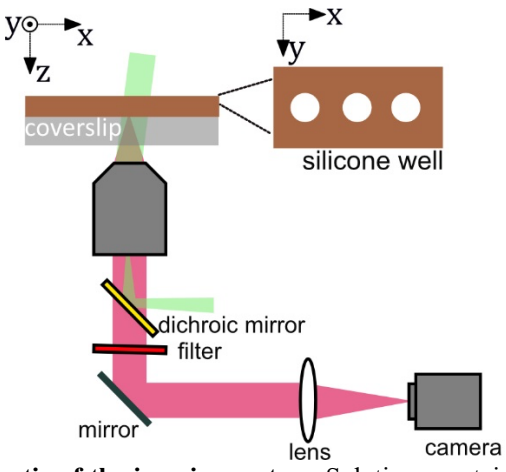
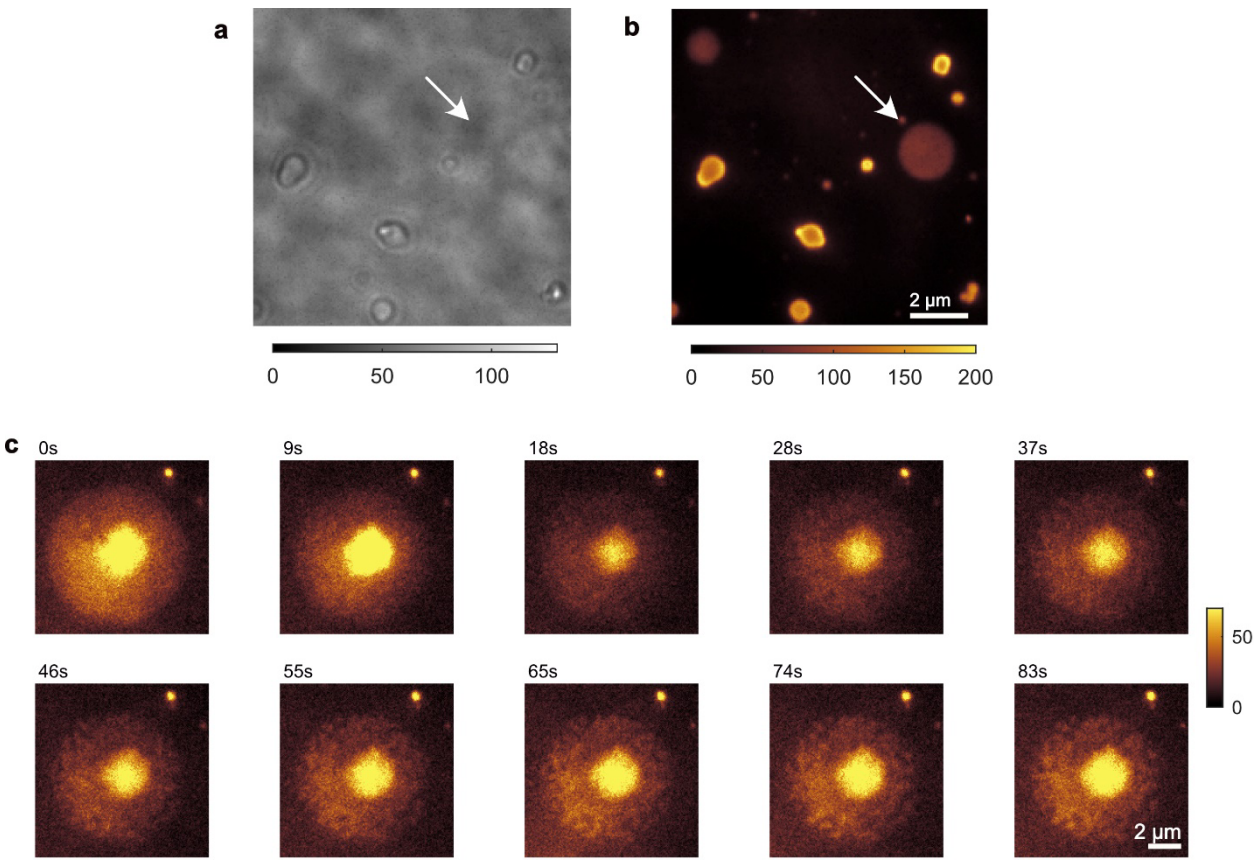
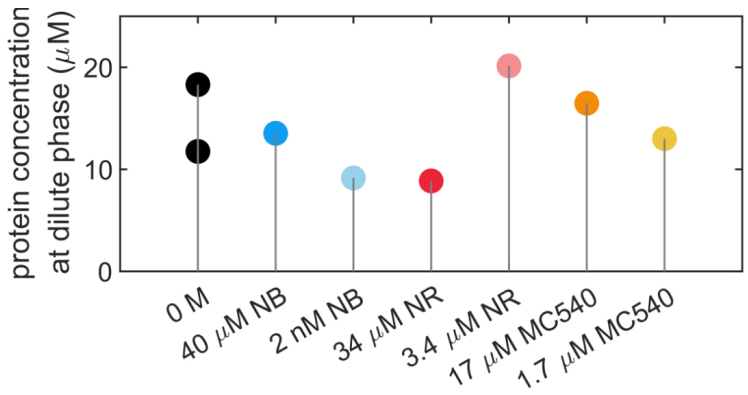


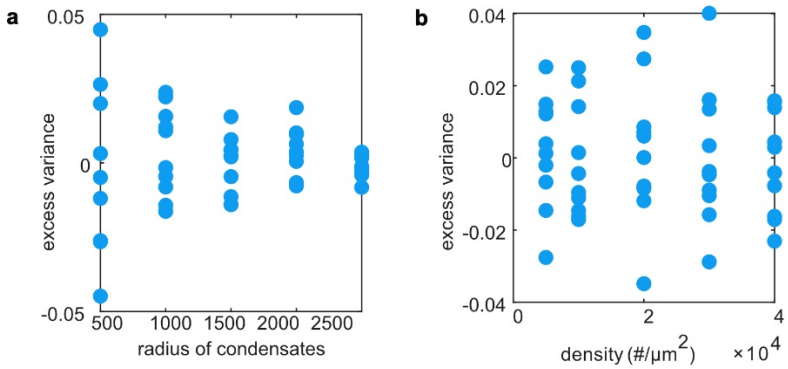
Extended Data Fig. 1. Schematic of the imaging system. Solutions containing the condensates are deposited in silicone wells adhered to a coverslip. An excitation laser is coupled into an objective lens to illuminate the sample. Fluorescence from fluorogenic probes is collected from the focal plane of the objective and filtered by a dichroic mirror and a bandpass filter. After passing through a tube lens, the fluorescence is captured by a camera.



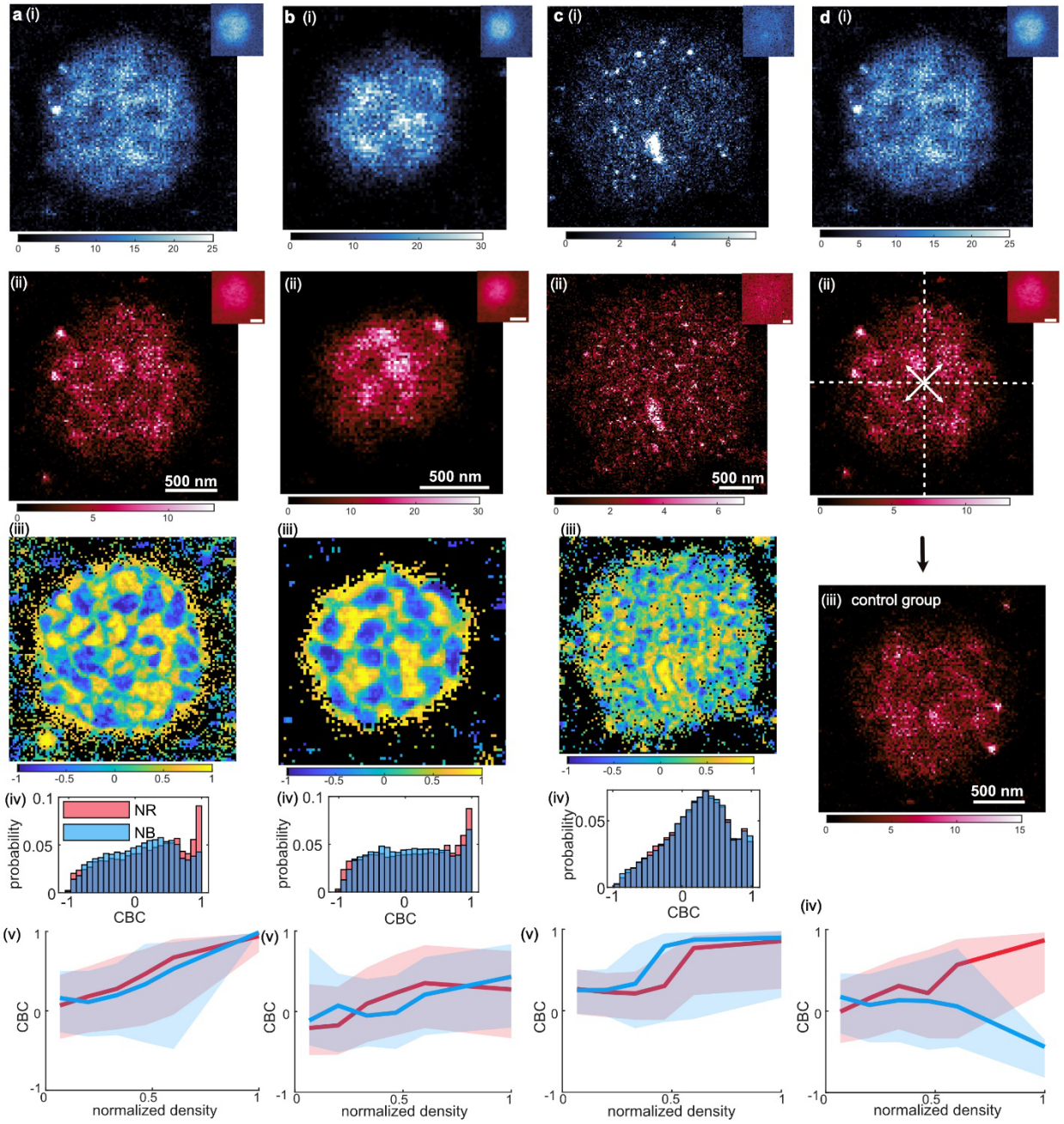
Extended Data Fig. 2. Imaging A1-LCD condensates using bright-field imaging and epifluorescence imaging of MC540. Unwetted condensates shown in **a**. have high contrast in the bright-field image. Images in **b**. show high MC540 fluorescence at their interfaces, while fully wetted condensates (arrow) show uniform MC540. **c**. A condensate imaged at different times using MC540.



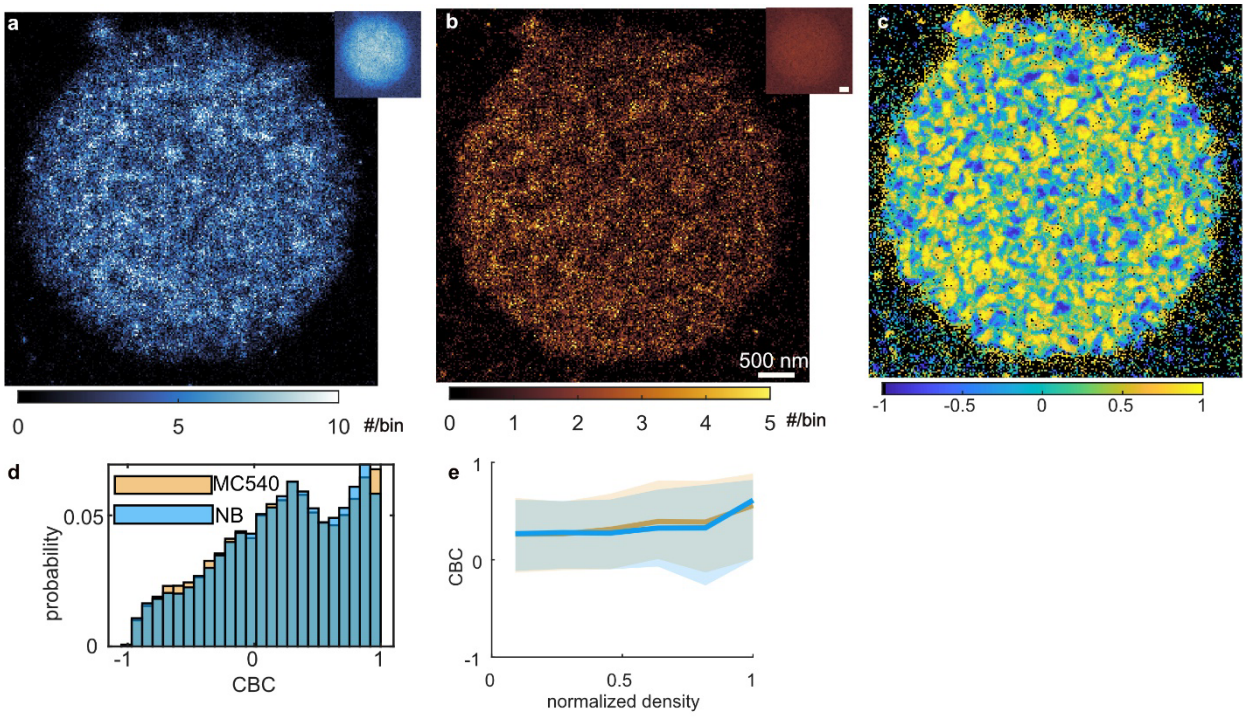
Extended Data Fig. 3. Fluorogenic probes have negligible influence on the driving forces for phase separation. The measured saturation concentrations of A1-LCD proteins change minimally, within the error of the measurements, upon adding NR, NB, and MC540 at different concentrations. Black circles are measurements made in the absence of dyes. The measurement is performed using ~ 90 μM A1-LCD in 20 mM HEPES buffer with 300 mM NaCl at 0 °C,



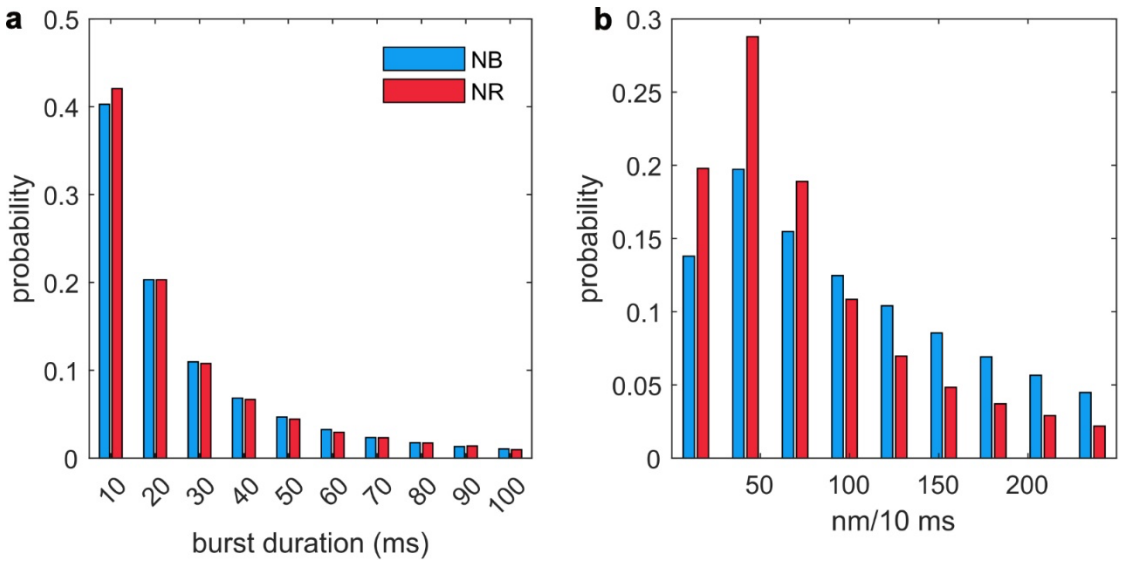
Extended Data Fig. 4. Excess variance is essentially zero for simulated homogeneous condensates with uniformly distributed localizations. Regardless of the condensates' (**a**) varying radius and (**b**) varying localization density, the excess variances are all close to zero. Ten simulations were performed for each condition.



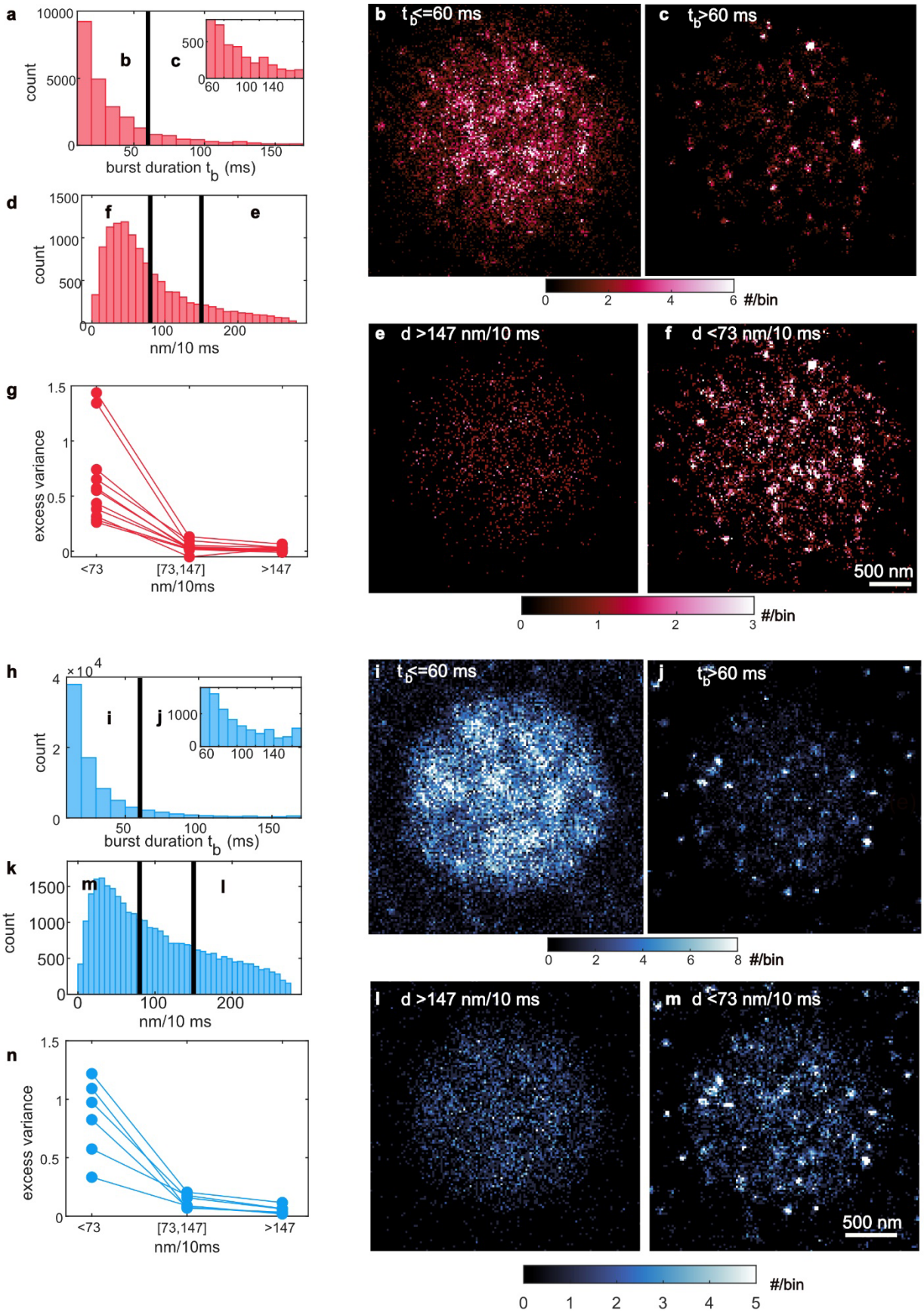
Extended Data Fig. 5. Spatial inhomogeneities within A1-LCD condensates imaged by NB and NR are highly correlated. a, b, and c, Correlation between NB, and NR for three condensates. Single-molecule localization microscopy images of single condensates collected using (i) NB and (ii) NR. Insets: epifluorescence images. Color bars: number of single molecules within each 20 nm × 20 nm bin. (iii) Coordinate-based correlation (CBC, see methods) between NR and NB localizations averaged for localizations within each 20 nm × 20 nm bin. (iv) Distribution of CBC scores for (blue) NB and (red) NR. (v) CBC of (blue) NB and (red) NR plotted as a function of localization density. Solid lines: median value; shaded area: 25th-75th percentile. **d.** A condensate measured using (i) NB and (ii) NR as shown in **a**. We swapped emitters in the four regions of (ii) to form (iii) a modified NR condensate that should have weak correlations with the original condensate in **a**. (iv) CBC values between (i) NB localizations and (iii) the modified NR localizations.



Extended Data Fig. 6. **Spatial inhomogeneities within A1-LCD condensates imaged by NB and MC540 are correlated with one another. a,b,** SMLM images of a condensate collected using **a**. NB and **b**. MC540. Color bars: number of single molecules within each 20 nm × 20 nm bin. Insets: epifluorescence images. **c.** Coordinate-based correlation (CBC, see methods) between NB and MC540 localizations averaged for localizations within each 20 nm × 20 nm bin. **d**, Distribution of CBC value for (blue) NB and (yellow) MC540. **e**, CBC of (blue) NB and (yellow) MC540 plotted as a function of localization density. Solid lines: median value; shaded area: 25^{th}-75^{th} percentile.



Extended Data Fig. 7. NB has longer burst durations and faster velocities than NR within A1-LCD condensates. Distribution of (**a**) burst duration and (**b**) magnitude of velocity for (blue) NB and (red) NR.


a
b
c
count
burst duration t_b (ms)
d
f
e
nm/10 ms
g
excess variance
<73
[73,147]
>147
nm/10ms
b t_b<=60 ms
c t_b>60 ms
#/bin
e d >147 nm/10 ms
f d <73 nm/10 ms
500 nm
#/bin
h
i
j
burst duration t_b (ms)
i t_b<=60 ms
j t_b>60 ms
#/bin
k
m
l
nm/10 ms
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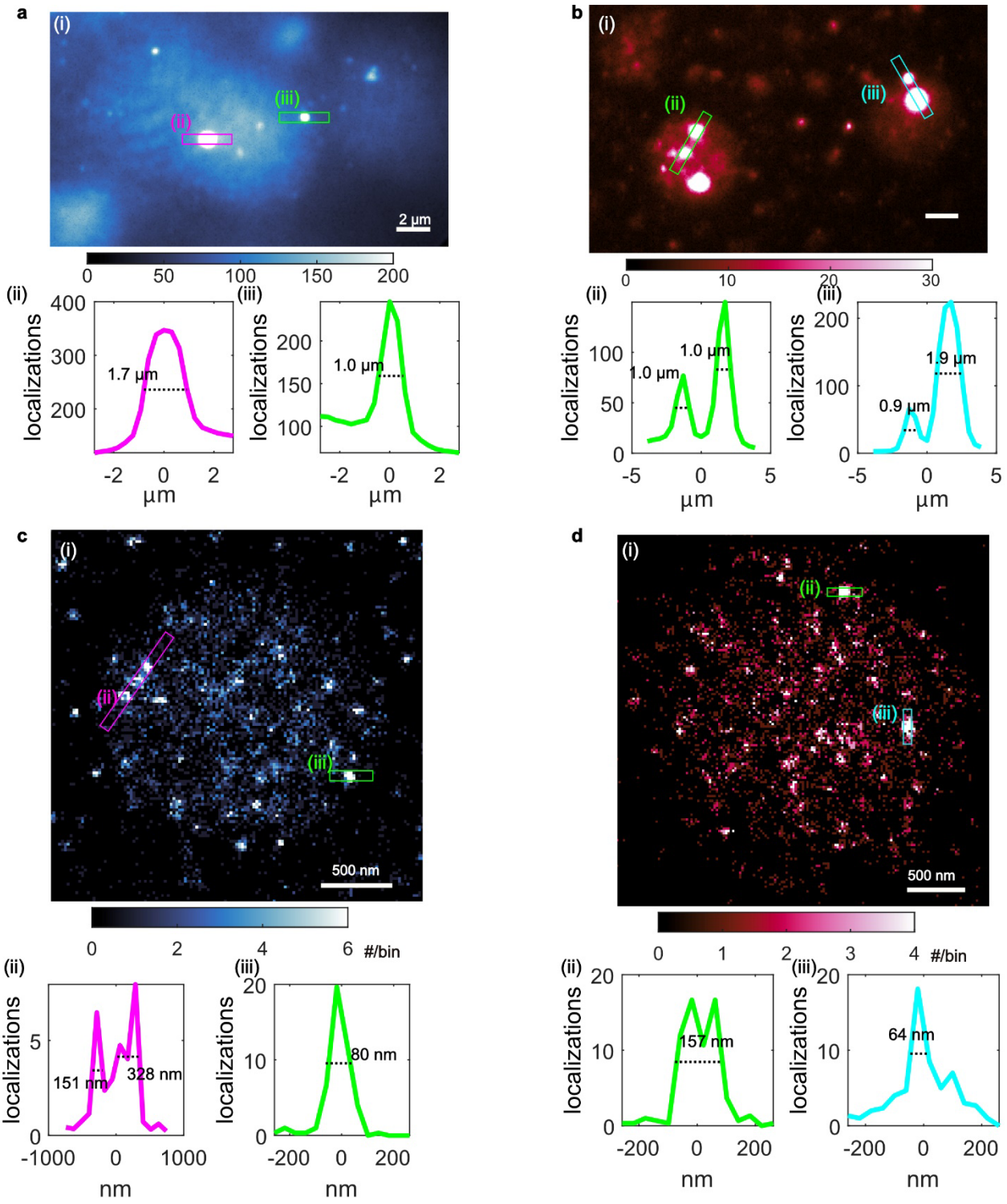
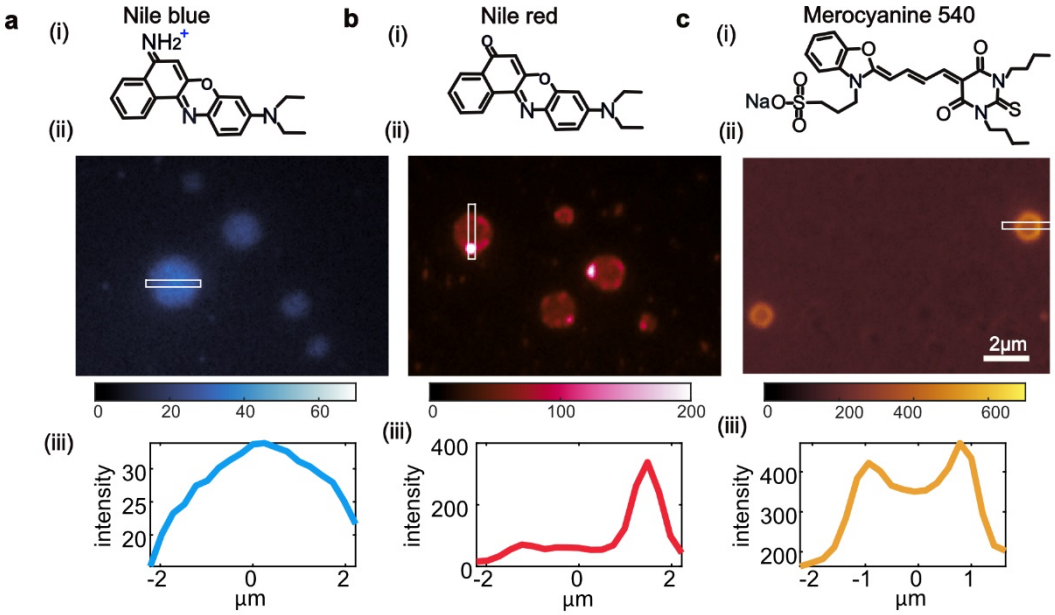
excess variance
<73
[73,147]
>147
nm/10ms
l d >147 nm/10 ms
m d <73 nm/10 ms
500 nm
#/bin

Extended Data Fig. 8. Tracking single-molecule fluorescence bursts and velocities reveals inhomogeneous molecular distributions within A1-LCD condensates. Single-molecule tracking of (**a-g**) NR and (**h-n**) NB. **a, h,** Fluorescence burst durations. **b, c, i, and j,** SMLM images of fluorogens with burst durations (**b, i**) shorter than 60 ms and (**c, j**) longer than 60 ms. **d, k,** Fluorogen velocities measured between consecutive camera frames (10 ms exposure time). **e, f, l, m,** SMLM images of molecules with magnitudes of velocities (**e, l**) larger than 147 nm/10-ms and (**f, m**) shorter than 73 nm/10-ms. **g, n**, Excess variance of localizations grouped by magnitude of velocity (left: ≤73 nm/10-ms, middle: between 73/10-ms and 147 nm/10-ms, right: >147 nm/10-ms). Lines: excess variance of molecules within each condensate.



Extended Data Fig. 9. Micro-scale and nano-scale hubs measured by epi-fluorescence imaging and SMLM. a,**b**, epifluorescence images of A1-LCD condensates using (**a**) NB and (**b**) NR, reproduced from Figs. 2(c,d). **c**,**d**, SMLM image of A1-LCD condensates using (**c**) NB and (**d**) NR, reproduced from Extended Data Fig. 8 (**f**,**m**). (ii,iii): intensity or localization profile along the long axis of boxes shown in (i) labelled with full-width at half-maximum.



Extended Data Fig. 10. Epifluorescence microscopy reveals that different fluorogenic probes sense chemical environments inside DDX4-IDR condensates from different perspectives. Imaging DDX4-IDR condensates using **a.** Nile blue (NB), **b.** Nile red (NR), and **c.** merocyanine 540 (MC540) (i) Chemical structures. (ii) Typical condensates as viewed by epifluorescence microscopy. (iii) Fluorescence intensity profiles along the long axis of white box shown in (ii).