USE OF FIDA FOR RAPID CHARACTERIZATION OF LIQUID-LIQUID PHASE SEPARATION

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- Rapid characterization of key parameters on a single experimental platform:
  - Dilute phase concentration
  - Relative droplet size distribution
  - Kinetics of droplet formation
  - Liquid to solid transitions
  - Affinity for LLPS modulating compounds
- Full automation and µL sample consumption
- No need for expert users

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INTRODUCTION

Liquid-Liquid Phase Separation (LLPS) is a phenomenon caused by the spontaneous and reversible formation of condensates that results in a highly concentrated dense phase and a dilute phase. [1]

In some cases, liquid to solid transitions occur, causing the formation of amyloid fibrils, amorphous aggregation, and gelation. Even though sometimes beneficial to the cell, these events are mostly associated with detrimental effects related to various neurological disorders such as ALS, Alzheimer's and Parkinson's disease.[5] As a result, LLPS has gained increased attention in academic and industrial setting.[6] Despite this, the field is lacking easily approachable methods for rapid characterization of the key parameters.

This Application Note is based on the paper of Stender, Ray & Norrild *et al.* published in Nature Communications.[3] It describes how FIDA is used as the new method to rapidly characterize multiple crucial LLPS parameters using µL of sample with no need of prior expertise in the technology.

Using Fida 1 as the single experimental platform, we measured dilute phase concentrations, droplet count, relative droplet size distribution, kinetics of droplet formation, maturation into amyloid fibrils as well as the affinity between proteins undergoing LLPS and LLPS-modulating compounds (Figure 1).

More specifically, we analysed the influence of ssDNA on the condensation of the n1 domain of human DEAD-box helicase 4 (Ddx4n1). Ddx4n1 is a protein involved in creating the nuage in egg and sperm cells and is well known for its role in partitioning polynucleotides.[7] We also present how FIDA is used to study the liquid to solid transition of α synuclein into Thioflavin T positive amyloid fibrils a process involved in Parkinson's disease showing the great potential of the technology for the study of LLPS-related neurological disorders.[8]

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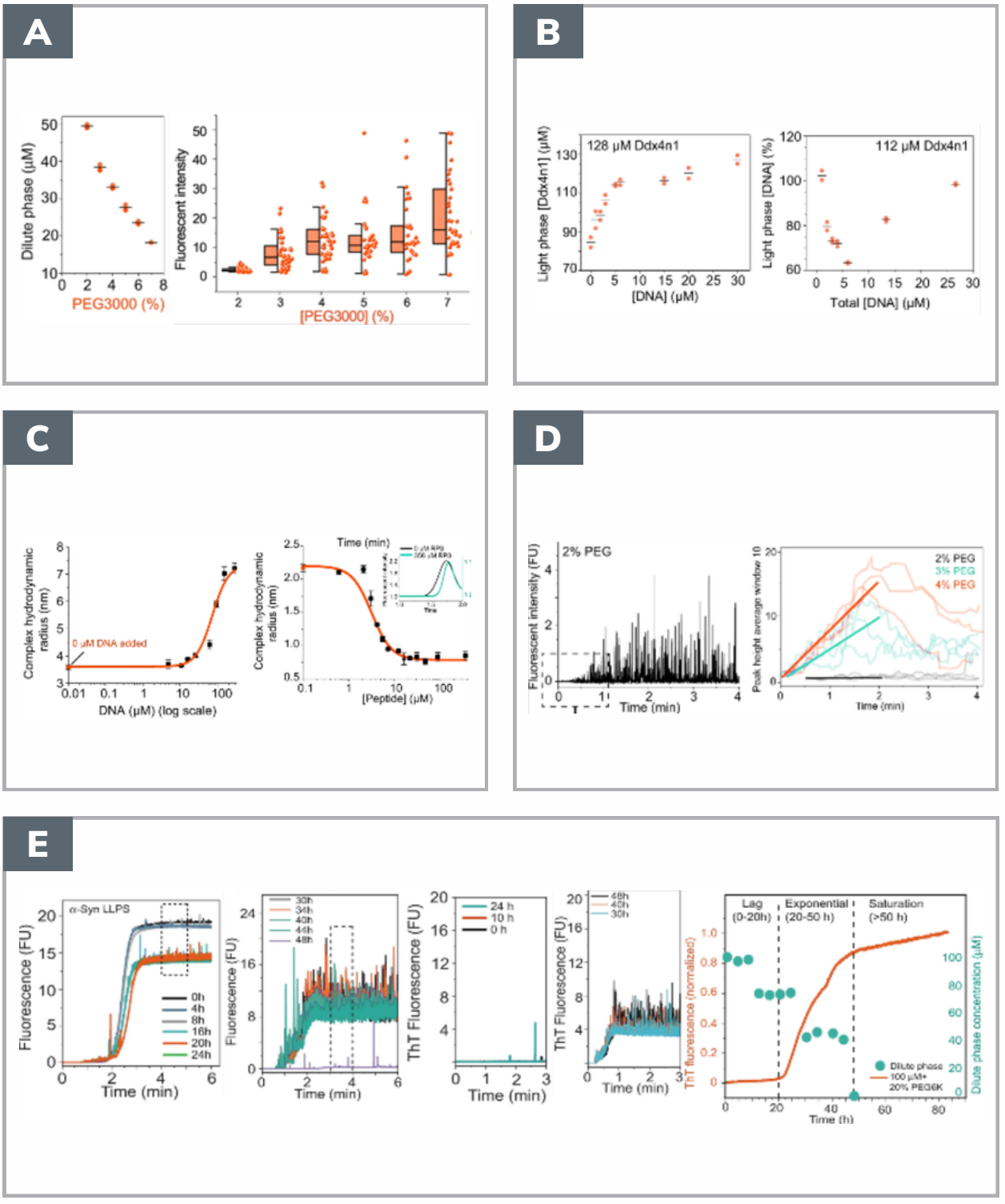

Figure 1. Overview of parameters characterised by FIDA technology. A. Dilute phase concentration, droplet count, relative droplet size distribution, B. Influence of LLPS modulating compounds, amount partitioned, re-entrant LLPS, C. Affinity of LLPS modulating compound, size of complex, conformational change, D. Droplet formation kinetics Ostwald ripening, E. Simultaneous monitoring of LLPS and subsequent amyloid aggregation. All this data is publicly available and can be found in Nature Communications.[3]

MATERIAL & METHODS

Fida 1 equipped with a 480 nm LED excitation detector. A standard or HS-coated Fida 1 capillary (L: 1m, ID, 75 µM Leff: 84 cm). Temperatures and buffers were system-specific. For most experiments the capillary was prefilled with buffer and the samples flowed through at a constant pressure. Trace amount of labelled biomolecules (<1 %) was used.

Initially, the samples were stored above the cloud point temperature in the autosampler and then flowed through the capillary which was kept at a temperature below the cloud point, causing LLPS (Figure 2A).The time interval between LLPS initiation and detection is the same for all samples eliminating potential bias caused by aging effects occurring at different times.

When a droplet passes the detector a signal spike is observed while the baseline of the top of the sigmoidal “spikograms” corresponds to the dilute phase concentration. The intensity of the droplet spikes relates to biomolecule concentration in the droplet, droplet size and the lateral position of the droplet in the capillary. In a phase diagram with horizontal tie lines, the distribution of signal spike intensities is directly related to the droplet size distribution.[3]

Detailed descriptions can be found in the experimental section of Stender, Ray & Norrild *et al.* [3]

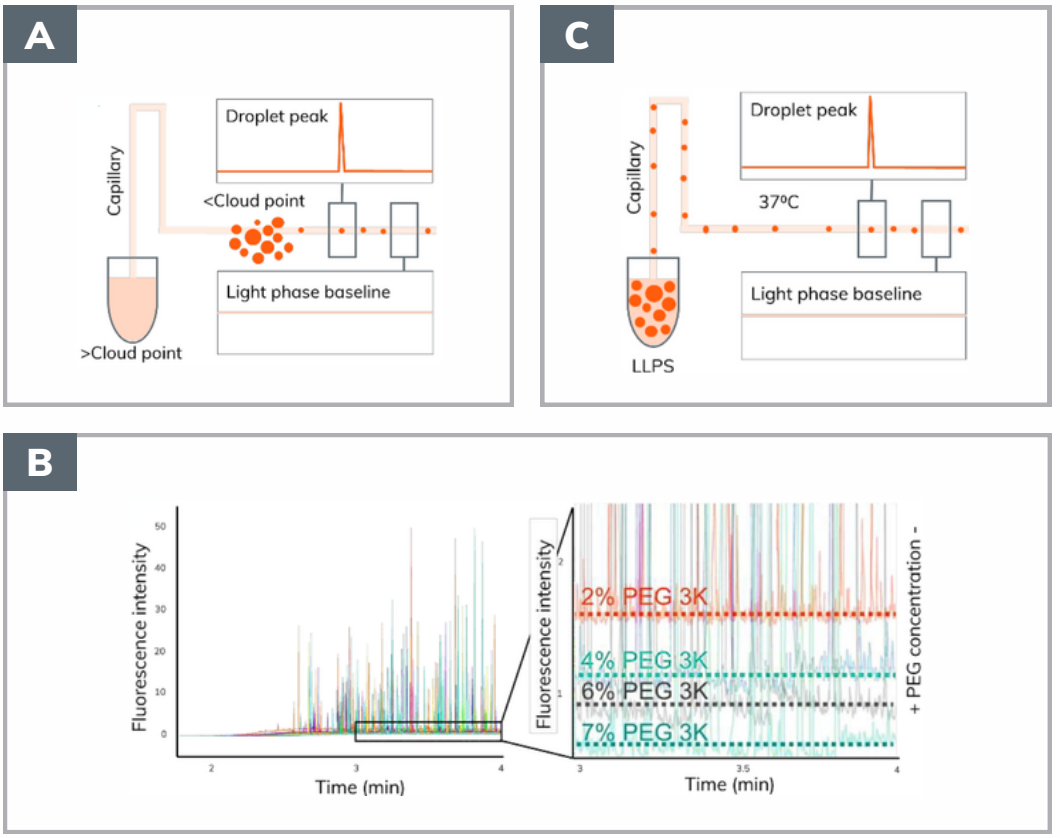

Figure 2. Example of FIDA LLPS analysis performed. **A.** The samples are kept in the autosampler above the cloud point. When injected into the capillary, kept at below cloud point temperature, LLPS occurs. **B.** When a droplet passes the detector a signal spike is observed. The baseline corresponds to the dilute phase concentration. **C.** If the cloud point is inaccessible, the samples can also be stored below the cloud point in the autosampler and injected into the capillary in condensed state.

RESULTS

Influence of ssDNA on LLPS of Ddx4n1

Human Ddx4n1 is well known to strongly partition nucleotides inside the protein droplets[7]. When no single-stranded DNA (ssDNA) is present, the Ddx4n1 dilute phase concentration is ~88 µM and several spikes are observed indicating that LLPS has occurred (Figure 3A). As the concentration of ssDNA increases, so does the dilute phase concentration of Ddx4n1, while the relative droplet size distribution decreases. Above 6 µM of ssDNA no droplets are detected. The dense phase is eventually completely dissolved at 30 µM ssDNA. This is surprising as Ddx4n1 strongly partitions DNA.[7]

To ensure that the ssDNA enters the droplets, the labelling strategy was switched using Alexa488-labelled ssDNA instead of YFP-labelled Ddx4n1 as a tracer (Figure 3B).

At 1 µM ssDNA, most of the ssDNA is found in the dilute phase but is also observed in the droplet signal spikes. At 6 µM ssDNA, its dilute phase concentration decreases down to ~60 %. However, the droplet size distribution decreases and the spikes eventually disappear at concentrations similar to the ones used in the first experimental design. As the concentration of ssDNA is further increased, eventually it is all found in the dilute phase and LLPS is completely abolished (Figure 3B). This indicates that the ssDNA is indeed partitioned in the droplets, but its presence is also detrimental to LLPS, indicating a potentially strong and disruptive interaction with Ddx4n1.

The interaction between Ddx4n1 and ssDNA was characterised by standard FIDA assay. The dissociation constant (KD) was found to be 50.9 ±11.1 µM (Figure 3C), revealing that the affinity is an order of magnitude above the concentration required to dissolve the droplets. This indicates that the detrimental effect of ssDNA on Ddx4n1 LLPS is not due to direct interaction in the dilute phase but rather multivalent interaction happening at high concentrations inside the droplets. This is further investigated and discussed by Stender, Ray & Norrild *et al.* [3]

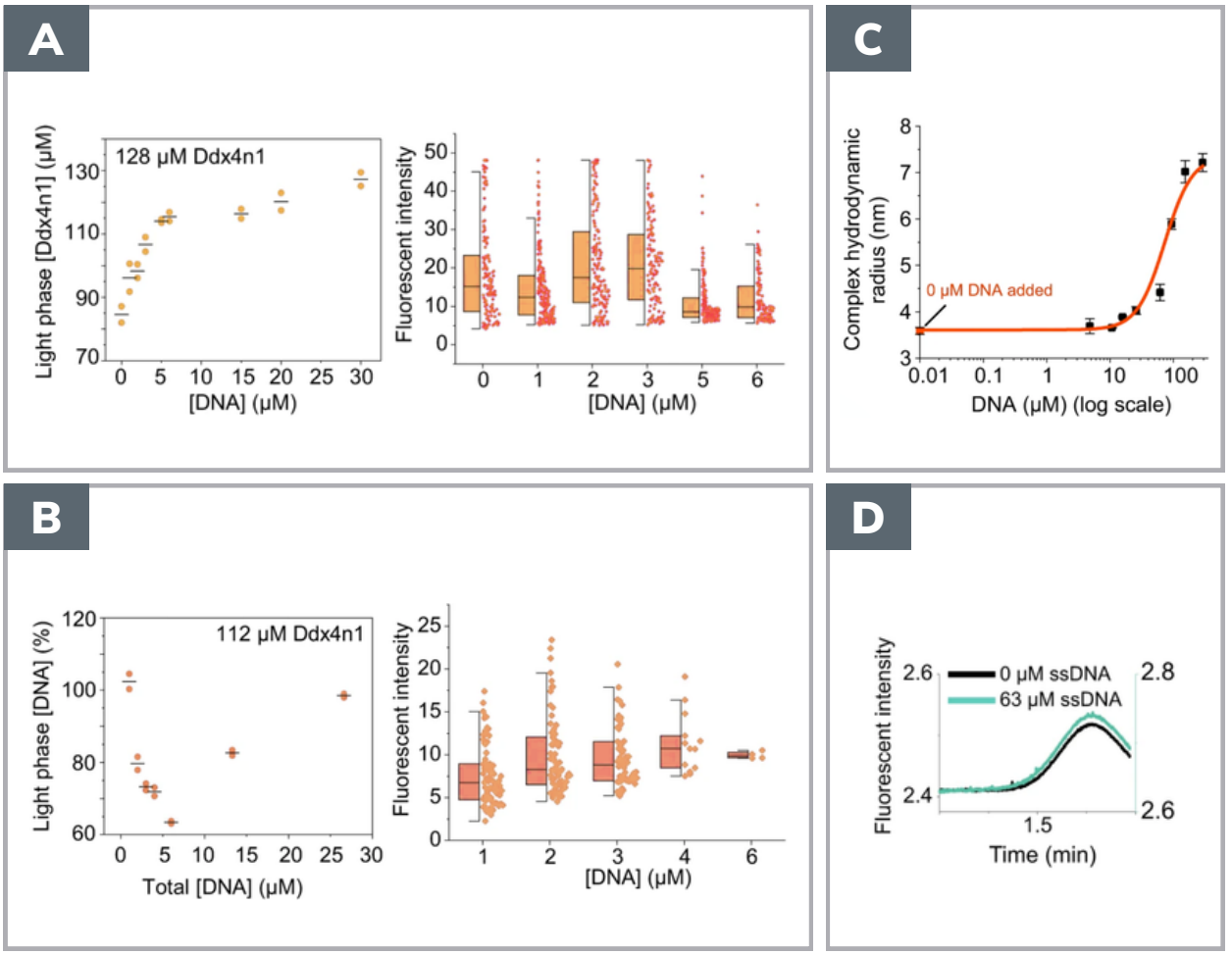

Figure 3. Characterization of the effect of ssDNA on Ddx4n1 LLPS. A. Dilute phase concentrations, droplet count and relative size distribution as a function of ssDNA concentration using YFP-labelled Ddx4n1 as tracer. B. Percentage of total ssDNA in the dilute phase, droplet count and relative size distributions as a function of total ssDNA using Alexa488-labelled ssDNA as tracer. C. Measured hydrodynamic radii of YFP-Ddx4n1 in complex with ssDNA. The red line is the fitted 1:2 bindingmodel. D. Overlayed Taylorgrams.

Aberrant liquid to solid transition of α-synuclein

The amyloid fibril formation of human α-synuclein (α-Syn) is observed in Parkinson's disease, while the protein was recently found to undergo LLPS under pathological conditions.[8]

Firstly, the LLPS of α-Syn was analysed (Figure 4A). The experiment was performed as described in Figure 2B. During the first 4 hours, no LLPS was observed as all protein remained in the dilute phase. At 8h, spikes were observed, and the dilute phase concentration slightly decreased indicating the initiation of droplet formation. After 16h, the concentration decreased further to ~70 µM and significant spikes were observed. The spikes disappeared when the sample was diluted, confirming that spike signal originates from liquid droplets (Figure 4C). After 30 hours, the dilute phase concentration further dropped to 40-50 µM and the spikes could no longer be dissolved by dilution indicating a liquid to solid transition of α-Syn. After 48h, only a low concentration of α-Syn remained in the dilute phase (Figure4B).

A simultaneous experiment was performed using Thioflavin T (ThT) as a tracer (Figure 4D). ThT is a dye specific for amyloid fibril formation. After 30h, when the spike signal became irreversible, a significant rise in the ThT signal was observed, indicating that the irreversibility of the droplets is caused by amyloid fibril formation. These results are further investigated and discussed by Stender, Ray & Norrild *et al.* [3]

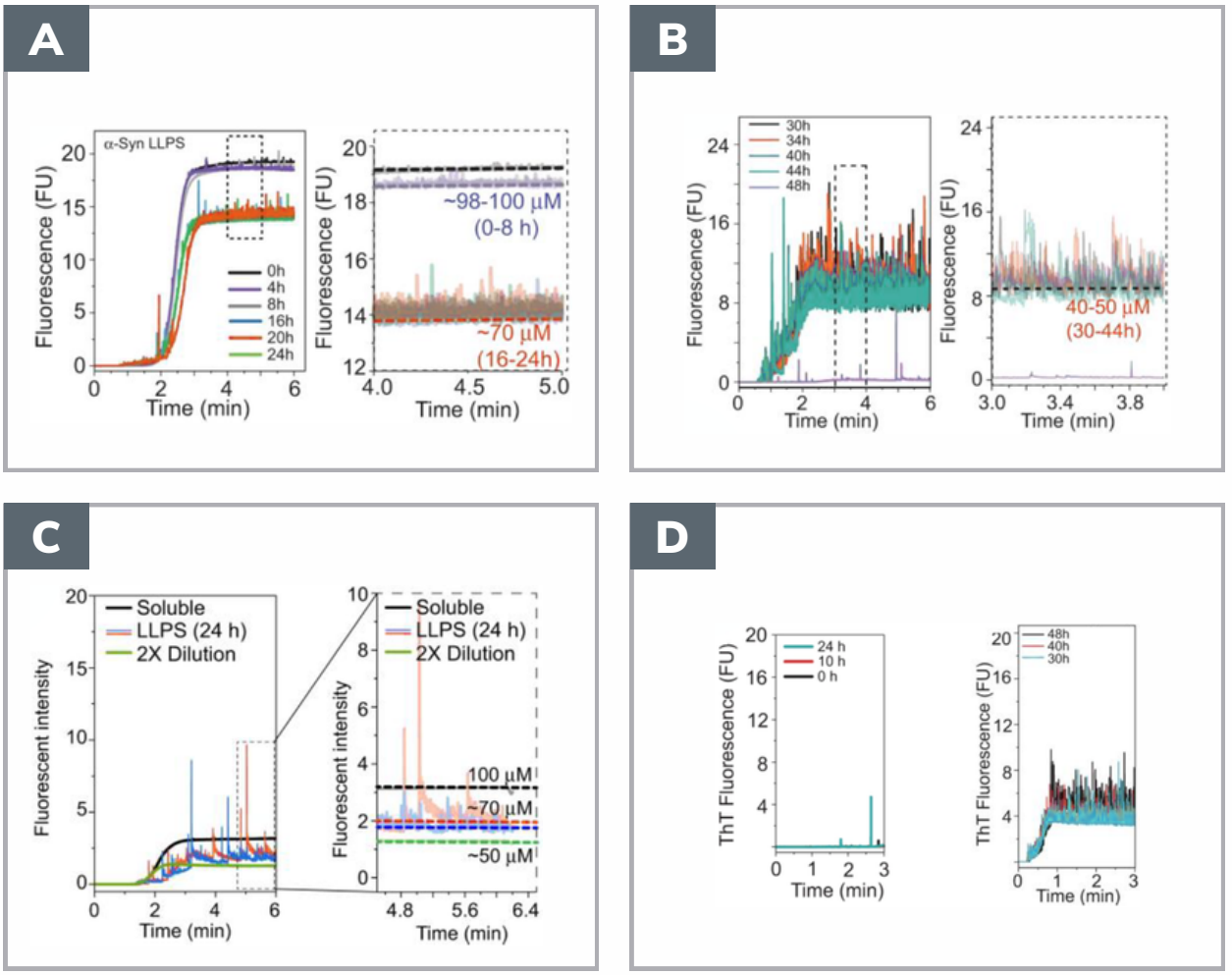

Figure 4. FIDA characterization of liquid to solid transition of α-Syn. **A.** First 24h incubation of α-Syn and the initial stage of LLPS, **B.** 30 – 48h of incubated α-Syn, **C.** The 24 h samples diluted; the disappearing spikes indicate droplet formation reversibility, **D.** The experiment described in A and B with ThT as tracer indicating amyloid fibril formation after 30h.

CONCLUSIONS

This scientific work demonstrates the versatility and strength of FIDA in LLPS analysis3. Using only µL of sample and without any need for expert users, we described the detrimental effects of ssDNA on the condensation of human Ddx4n1. We also described the LLPS of α-synuclein and the aberrant liquid to solid transition into amyloid fibrils. The results presented in this App Note and much more are described and discussed in detail in Nature Communications.[3]

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