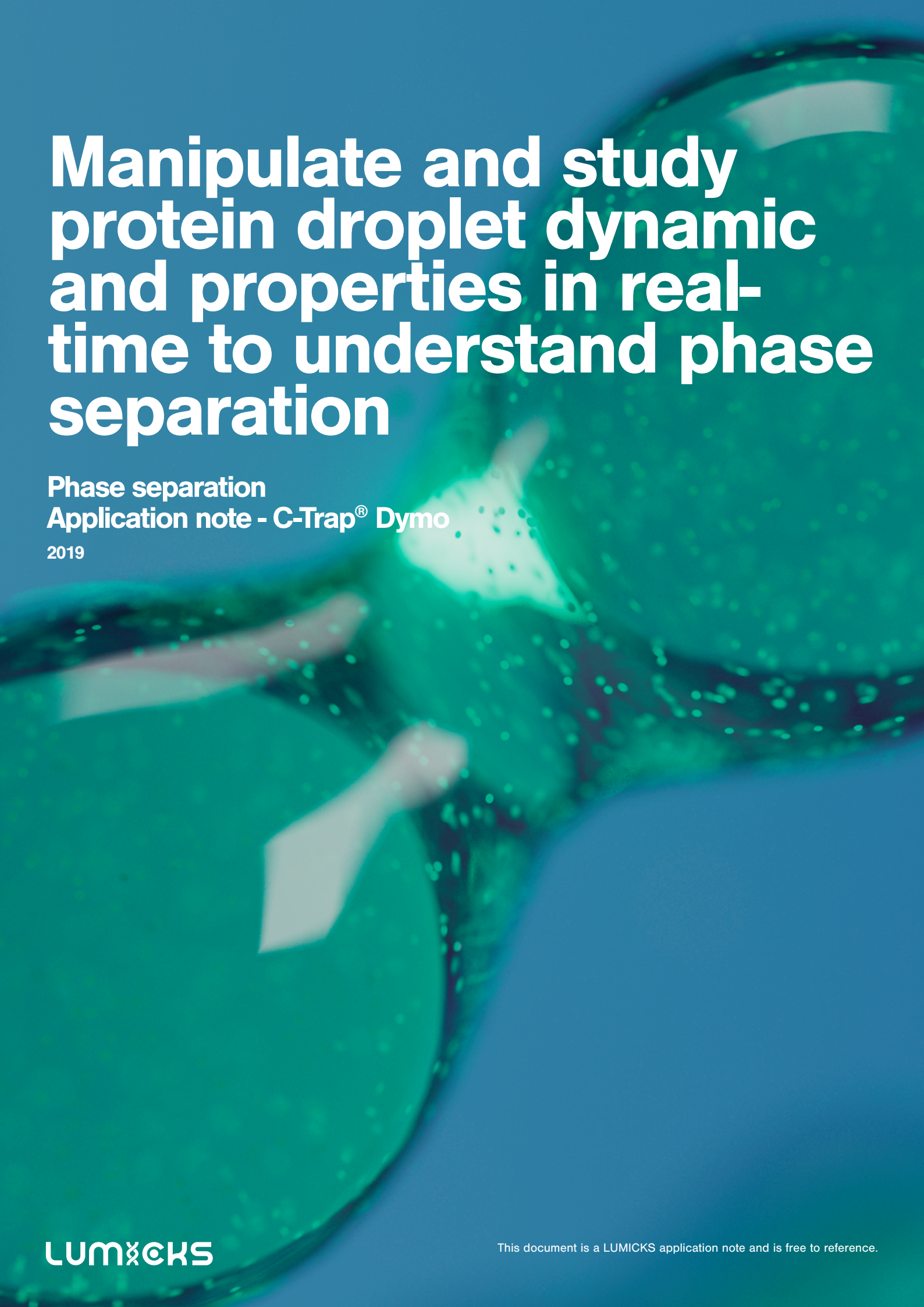
Manipulate and study protein droplet dynamic and properties in real-time to understand phase separation

Phase separation
Application note - C-Trap® Dymo

2019



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Mounting evidence suggests that membrane-less organelles in cells are semi-transient structures, formed to enable efficient interactions between specific biomolecules. Understanding the formation of these structures, their physical properties, and mechano-chemical interactions have aided our understanding of several cellular processes. On top of that, the link between droplet formation and medical conditions, such as neurodegenerative amyotrophic lateral sclerosis (ALS) and cancer, has enhanced the need for appropriate assays that can assess related properties. In these cases, proteins can aggregate to promote a successive solidification of droplets, forming gel-like or irreversible solid structures known as amyloid fibrils (plaques).

Membrane-less organelles and other subcellular structures result from liquid–liquid phase separation of proteins and RNA. These include stress granules, nucleoli, RNA-transport granules, and possibly heterochromatin formation. Studies have (since the 1930s) progressively contested the conventional notion that the protein machinery is homogeneously distributed in the cytoplasm – like a soup of soluble molecules and membranous organelles. Instead, they suggest a formation of structures through multivalent interactions associated with specific protein regions.

While we have come a long way in understanding such processes, these droplets are extremely dynamic, dissolving and forming in response to cues that we do not yet fully understand. As a result, the proponents of liquid–liquid phase separation have been unable to capture the dynamics and properties of these droplets with the current techniques. There is, in other words, a need to develop tools and systematic experiments that can unravel the fundamental processes of phase separation:

- Which factors regulate liquid protein droplets and their transitions to solid aggregates?
- What are the relationships between liquid protein droplets and the irreversible solid amyloid fibril structures?
- What are the material properties of protein droplets during solidification?

In this application note

This application note introduces you to a new approach that correlates optical tweezers and the latest imaging techniques to study phase separation properties in real time. Based on four recent publications in the field, we show you how you can use the C-Trap® Dymo to investigate the assembly and disassembly of droplets and the properties that can lead to the pathological solidification of these structures.

Features of the C-Trap Dymo

The C-Trap Dymo combines optical tweezers, state of the art imaging, and an advanced microfluidics system to capture, manipulate, and visualize droplet structure properties at different conditions.

The optical tweezers enable you to trap micron-sized particles, such as protein droplets or beads, with a highly focused laser beam. You can then manipulate the position of the trapped structure by controlling the direction and force of the beam.

The real-time imaging lets you visualize the trapped droplets and the associated biomolecules as you manipulate the droplet structures. For example, you can label different biomolecules involved in droplet processes and follow their trajectory using the fluorescence microscopy system.

The microfluidics system improves the experimental workflow and helps you to add reagents to the system in separate channels. No physical barriers separate the highly stable flows, allowing you to control and navigate the different reagents.

Challenges and solutions

The challenges

Although rapidly progressing, liquid–liquid phase separation is a new field and thus has a limited repertoire of assays able to assess its associated kinetics. On top of that, the processes of liquid–liquid phase separation and its link to diseases may be highly context dependent, relevant only to certain conditions, and hence possibly rare in the cellular environment. These uncertainties naturally limit our ability to connect *in vitro* results to physiologically relevant processes.

Nevertheless, advancements in the field have paved the way for a deeper understanding of the relevance of protein droplet formation, condensation, and aggregation.

Still, to date, most of the assays used to study the assembly or disassembly of protein droplets and their properties have relied on spontaneous events or binary readouts.

For example, classic liquid droplet fusion relies on the spontaneous interactions between droplets. Importantly, this method merely gives a binary readout (the droplet fuses or not) and does not offer a means of comparing fusion properties (for example, through time to droplet relaxation).

Another frequently used assay is the fluorescence recovery after photobleaching (FRAP) approach that characterizes the mobility of droplet molecules by, as the name suggests, their ability to recover photobleaching. While the method serves well to compare two-dimensional lateral diffusion, it fails to show the three-dimensional properties of the measured droplets, for example, the viscoelasticity.

Other methods like rheology measurements through cone plate rheometers require high volumes of reagents, which make them less relevant for understanding biological processes and are, on top of that, costly.

The solutions

The C-Trap® Dymo offers key functions that allow you to measure subtle droplet properties and compare the results between different conditions. The high spatiotemporal resolution of the instrument enables you to detect specific droplet events while you force-manipulate the substrates. It extends phase separation and droplet analysis by providing you three main functions:

1. Optical tweezers that can manipulate the reaction you are analyzing.
2. Fluorescence microscopy that allows you to follow the reactions in real time.
3. An advanced microfluidics system that speeds up your workflow and allows you to reconstruct your assay in physiologically relevant conditions.

All-in-all, with the C-Trap Dymo it is possible to:

- Control your assays by force-inducing droplet fusion.
- Detect subtle differences by comparing time-to-relaxation between droplets in different conditions.
- Assess viscoelastic properties inside the structure through microrheological measurements.
- Compare different conditions by evaluating the properties of single droplet-structures.

Models and conditions

Here, we present the two approaches the studies used to assess specific properties of protein droplets through applied forces, namely:

1. Droplet Fusion, and
2. Microrheology and Viscocity.

Each of the presented experiments in these studies used one of three RNA-binding proteins as models to understand droplet properties in different conditions:

- Heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) – mutation variants have been implicated in amyotrophic lateral sclerosis (ALS), among others.
- Fused in Sarcoma (FUS) – mutations implicated in neurodegenerative diseases, such as ALS.
- Guanyl-specific ribonuclease PGL-3 – involved in forming P-granules, perinuclear RNA granules in the germline of the nematode worm Caenorhabditis elegans.

Each study exposed the models to different conditions that induce changes in protein droplet properties and structures:

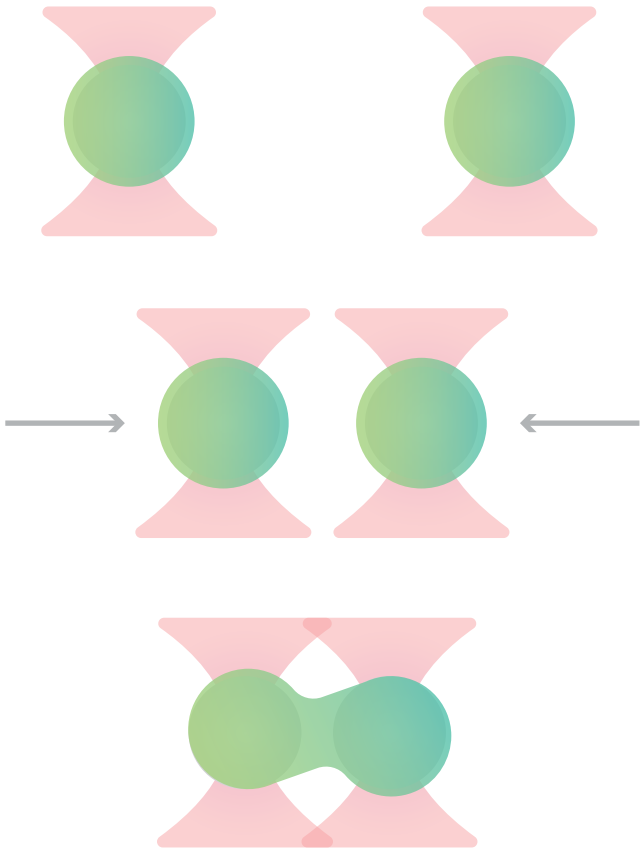
- Varying concentrations of molecular crowders.
- Varying ratios of RNA to ribonucleoproteins.
- Missense mutation of hnRNPA1.
- Varying salt concentrations.

Droplet fusion

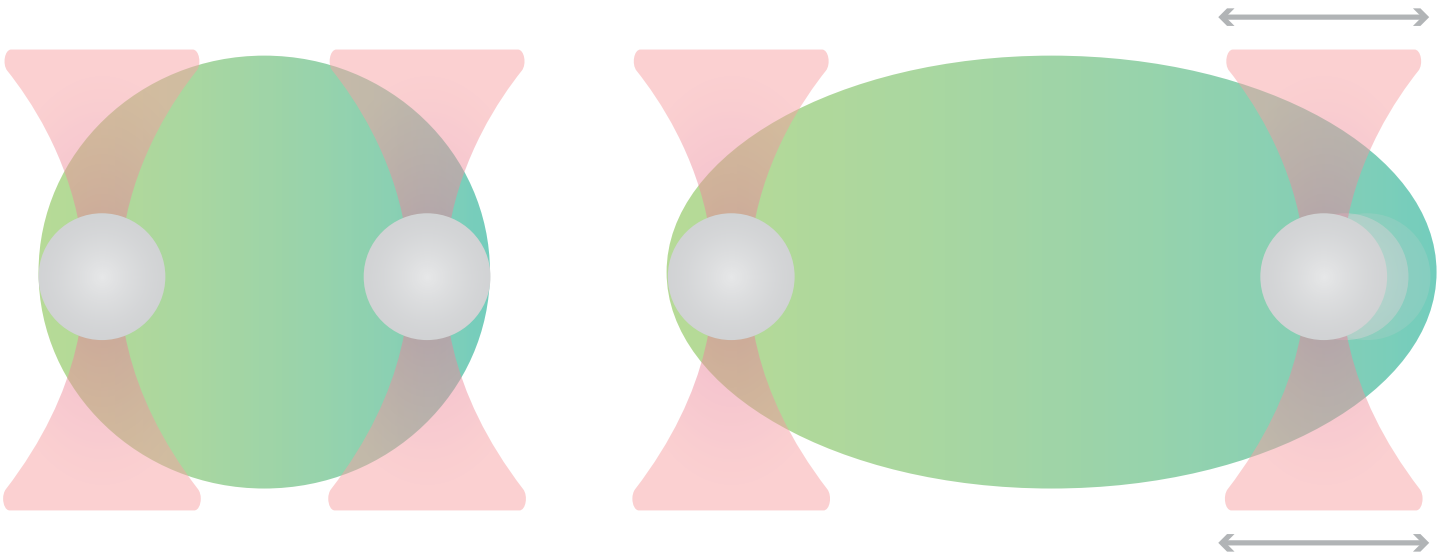
Researchers can use correlated optical tweezers and fluorescence microscopy to study and compare the rigidity of protein droplets at different conditions. By trapping two droplets with a dual trap they can direct the structures into proximity. Measurements show the time required for two droplets to condense into one larger droplet **(Figure 1)**. The measured time-to-relaxation indicates the stability or rigidity of the assessed protein droplets.

Microrheology and viscosity

Besides directly trapping droplets, researchers can also trap different types of micron-sized particles with optical traps, for example polystyrene beads. A dual trap-setup can capture two beads and bring them into adhesive contact with a droplet **(Figure 2)**. While holding one of the beads in place, the second trap moves the other bead back and forth along the x-axis, thereby deforming the droplet. The force-induced oscillations of the bead reveal the required force and amplitude needed to move the bead within a specific region along the x-axis of the droplet. Force differences at different conditions serve to estimate changes in droplet stiffness and viscous drag, which is dependent on the friction.



1 Optical tweezers are used to trap two fluorescently-labeled protein droplets and bring them into close proximity with each other to induce their fusion.



2 A schematic illustration depicting an optical trap-based microrheology assay. Optical tweezers are used to trap two polystyrene beads that are next brought into adhesive contact with a fluorescently labeled protein droplet. Once in contact, one of the beads is manipulated to move along the x-axis, thus probing the microrheological properties of the droplet.

Experiments

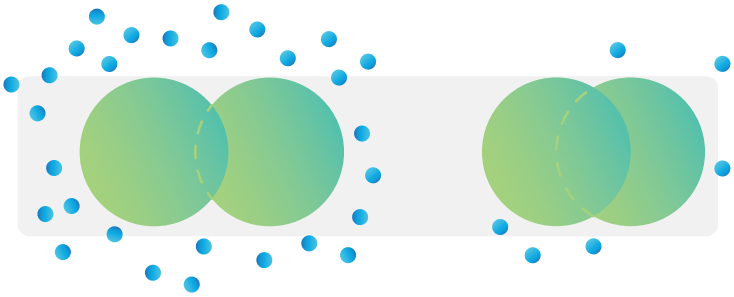
Measuring the effects of molecular crowders on droplet fusion.

The background

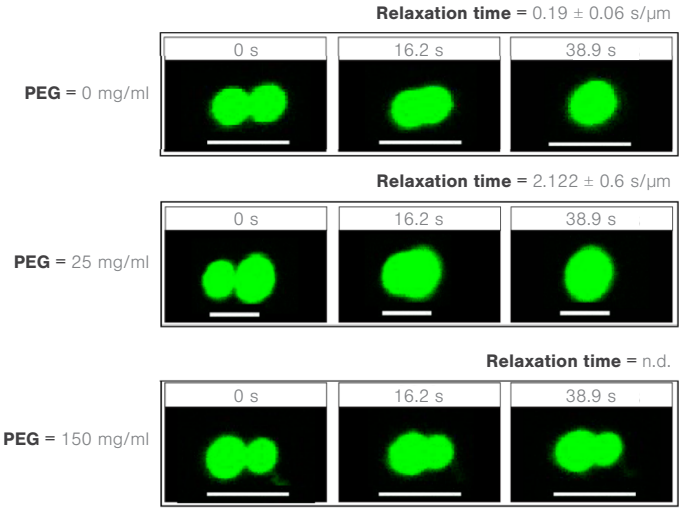
How do different concentrations of molecular crowders affect the coalescence of protein droplets?

The cellular environment contains several macromolecules that can change the molecular and structural properties of the cell in a concentration-dependent manner. The dynamics of molecular crowding can, in part, be explained by an excluded volume-effect, which means that a space occupied by one molecule becomes inaccessible for another.

These changes influence the formation and properties of membrane-less bodies and can directly or indirectly affect cellular processes, such as protein conformation, RNA folding, or protein organization. Apart from standard functions, molecular crowders may also affect the development of condensates and the formation of fiber-like structures associated with several neurodegenerative diseases.



3 A schematic illustration representing the impact of molecular crowder concentrations on the fusion of protein droplets. Here, a higher concentration of crowders implicates the fusion of the two droplets compared with lower concentrations.



4 Images showing time-lapses of FUS droplet fusion as a function of PEG8000 molecular crowder concentrations. Droplet fusion was induced using optical tweezers, bringing the droplets into proximity at constant velocities in the presence of 0, 25, or 150 mg/ml PEG8000. Credit: Kaur, T. et al., Biomolecules, 19 February, 2019. This work is licensed under CC BY-4.0 (https://creativecommons.org/licenses/by/4.0/).

The experiment

To understand how molecular crowding affects the phase behavior of the RNA-binding protein FUS **(Figure 3)**, researchers from the **Banerjee Lab at the University at Buffalo**, used the polyethylene glycol PEG8000 as model molecular crowder. By altering the levels of PEG8000 (up to 150 mg/mL), the investigators could evaluate the contribution of crowder-concentrations in FUS condensation.

The researchers first trapped two FUS droplets using optical tweezers. They next moved one trapped droplet towards the other one at a constant velocity, and measured the time to fusion (time-to-relaxation) at different crowder concentrations. While the absence of PEG8000 crowders allowed for fast merging between the liquid FUS droplets (about 200 ms/µm), the presence of 150 mg/ml PEG8000 almost arrested condensation **(Figure 4)**.

The results indicate that depletion forces by molecular crowders, such as PEG8000, can regulate the viscosity of protein droplets. Hence, a crowded cellular environment can induce the solidification of RNPs, such as FUS, subsequently driving the droplets from a liquid-like to gel-like state.

Application ideas

Staying ahead of the curve

- Trap protein droplets and study their phase as a function of time, environment (for example, ionic strength), or concentration.
- Tether DNA or microtubules to study the effect of the droplets on these molecules, or the effect of molecules on the droplets.

Experiments

Measuring the effect of RNA/RNP molecular interactions on the material state and fusion of protein droplets.

The background

How do protein droplets stay separated from one another?

Previous ***in vitro*** experiments have shown that RNA molecules can inhibit the condensation between protein droplets at high RNA-to-Ribonucleoproteins (RNA-to-RNP) ratios[1]. By contrast, low RNA-to-RNP ratios can promote the same process, indicating RNA regulates droplet properties. Studies have suggested that RNA-binding to specific domains rich in positively charged arginine and glycine (R/G) amino acids – frequently found in RNPs – has a regulatory effect on condensate formation.

The experiment

Scientists from the **Banerjee Lab at the University at Buffalo** investigated the condensation properties between different RNAs and polypeptides. By comparing different combinations between purine-rich poly(A) or pyrimidine-rich poly(U) and arginine/glycine-rich (R/G-rich) or lysine/glycine-rich (K/G-rich) peptide domains they could investigate molecule-dependent condensation properties (Table below).

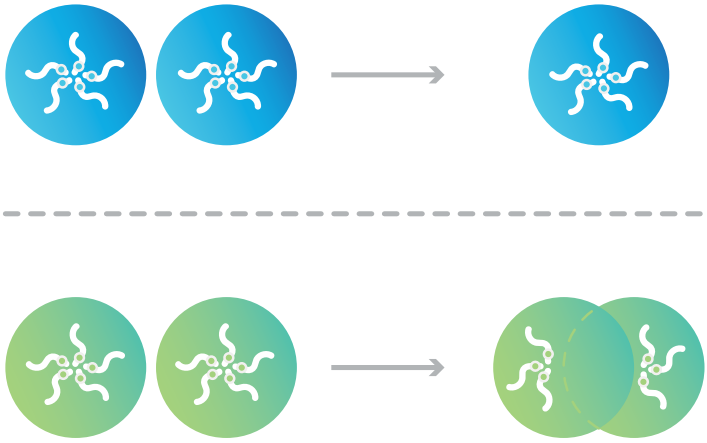
Table 1 Combination of the tested RNA-RNP complexes

Combo	RNA	Peptide
1.	poly(A)	R/G-rich domain $[RGRGG]_5$
2.	poly(U)	R/G-rich domain $[RGRGG]_5$
3.	poly(A)	R/G-rich domain $[KGKGG]_5$
4.	poly(U)	K/G-rich doman $[KGKGG]_5$

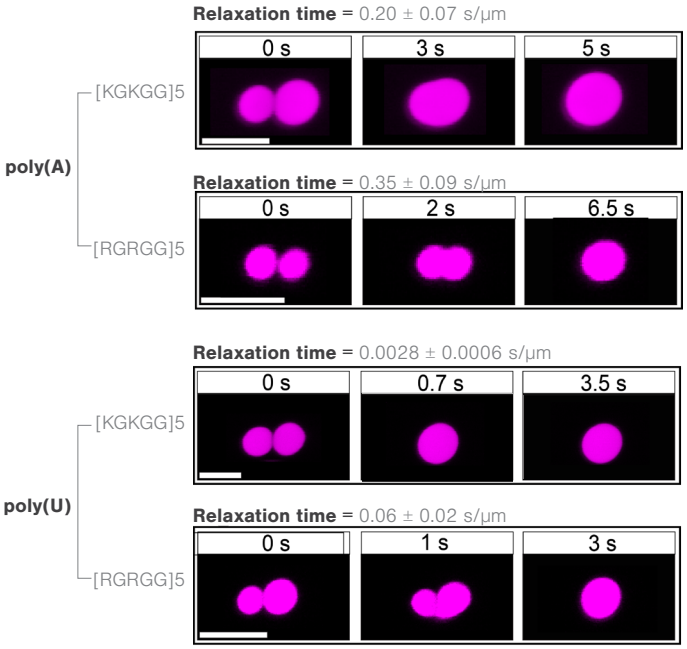
Of note, both peptide sequences ($[RGRGG]_5$ and $[KGKGG]_5$) contained the same charge at pH 7.5, namely +10e, ensuring similar interactions with the RNA molecules.

Using the C-Trap, the team compared the state of the respective condensates through force-induced droplet fusion (see Experimental design above), which serves to assess the corresponding fusion capacity and droplet stability through time-to-droplet relaxation. K/G-rich peptides complexing with poly(U) fused twice as fast (mean, 0.0028 s/µm) than R/G-rich peptides complexing with poly(A) RNA (Figures 5 and 6). The results indicate a higher fluidity and lower viscosity in the former and suggest that short-range attractions and long-range forces regulate the dynamics of RNA–peptide condensate formation, including coalescence and rigidity. The researchers further confirmed the observed sequence-specific phase properties using R/G-rich domains from a naturally occurring FUS model system in the presence of either poly(A) or poly(U) RNA.

Accordingly, the gathered findings can serve to understand mechanisms underlying organelle autonomy as well as pathological aggregations. The results suggest that sequences or structures of RNA and protein play a defining role in droplet dynamics and rigidity.



5 A schematic illustration representing the different times to relaxations upon droplet fusion depending on the specific RNA-RNP combination (depicted by the blue and green colors).



6 Time-lapse images comparing the times to relaxation of different RNA-RNP complexes during optical trap-induced droplet fusion. RNA-RNP complexes containing the R/G-rich domains showed slower relaxation compared with complexes containing the K/G-rich domain. Poly(A) RNAs also contributed to slower relaxation times compared with poly(U) RNAs. Data courtesy: Dr. Priya Banerjee.

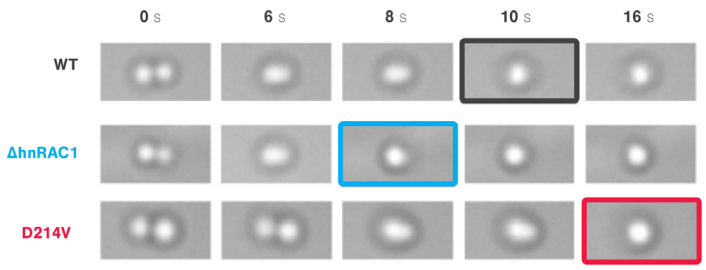
Experiments

Testing how variations in low-complexity domains influence the solidification of protein droplets.

The background

What influences the transition between liquid-like and solid protein droplets?

Many peptides or proteins involved in phase separation contain low-complexity domains that induce liquid–liquid phase separation through ionic interactions. These domains – characterized by an overrepresentation of specific amino acids compared with the proteome – are also prone to form protein and amyloid aggregates, and many neurodegenerative diseases contain missense mutations in the domains. Recent evidence has suggested that intermediate metastable and reversible amyloid fibrils are involved in the transition between liquid-like droplets and irreversible solid aggregates[2]. Here, researchers from the **lab of Dr. Bo Sun at ShanghaiTech** assessed how variations in low-complexity domains influence droplet solidification.



7 Time-lapse images comparing the times to relaxation of protein droplets consisting of wild-type (WT), RAC-depleted (ΔhnRAC1), or RAC-mutated (D214V) during optical tweezer-induced droplet fusion. Highlighted images indicate the time point of droplet fusion. Credit: X. Gui et al, Nature Communications, 1 May, 2019. This work is licensed under CC BY-4.0 (https://creativecommons.org/licenses/by/4.0/).

The experiment

The investigators first identified amyloid-forming low-complexity domain cores within the hnRNPA1 proteins called reversible amyloid core (RAC). These structures contain negatively charged aspartic acid, form hydrogels at a low temperature (4°C) and dissociate the formed fibrils at a higher temperature (25 °C).

Similar to the second experiment we show earlier, the investigators used force-induced droplet fusion to evaluate droplet properties. In this case, the researchers investigated the influence of hnRAC variants on the physical properties of hnRNPA1 droplets. Compared with wild-type hnRNPA1 droplets, the deletion of hnRAC1, a specific segment within the protein, resulted in faster droplet fusion (10 seconds and 8 seconds, respectively). Conversely, hnRAC1 missense mutation of the aspartic acid to valine (D214V) – associated with irreversible amyloid formation – resulted in an even slower droplet fusion (16 seconds; **Figure 7**).

Together with complementing assays, the researchers could establish that reversible amyloid fibrils reduce the fluidity and solidify protein droplets, which in turn, enhance phase separation. The findings highlight the role of reversible amyloids in the formation of stress granules and as an intermediate of irreversible fiber aggregates.

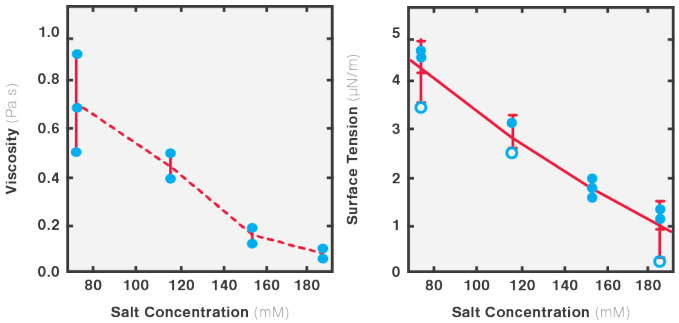
Experiments

Assessing the microrheology, viscosity, and elasticity of protein droplets upon increasing salt concentrations.

The background

What are the physicochemical properties of protein droplets?

Compartmentalization through liquid–liquid phase separation often depends on scaffolding proteins that form the base for the structure formation and colocalization of other biomolecules, including RNAs and proteins. The degree to which these biomolecules interact ultimately affects the material properties of the droplets, including droplet viscosity and elasticity, subsequently affecting their functions, for example, diffusion or structural rigidity.



8 Plots representing changes in viscosity (left) and surface tension (right) as a function of increasing salt concentrations. Adapted from Jawerth et al., Physical Review Letters, 18 December 2018.

The experiment

To understand the material properties and rheology of protein droplets, researchers determined the microrheology of PGL-3 protein droplets as a function of salt concentration.

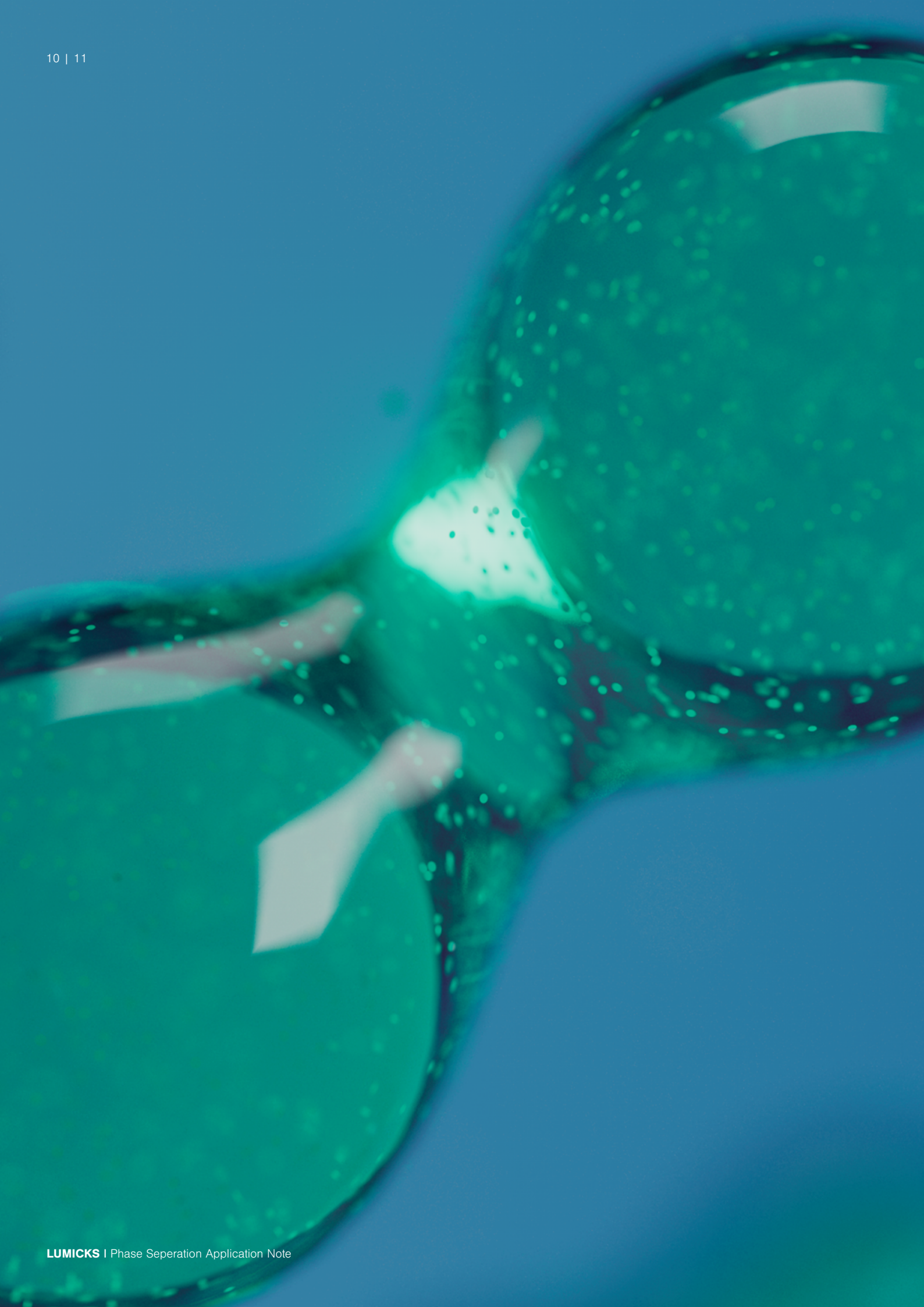
Using two optical traps, scientists from the **Hyman Lab at the Max Planck Institute of Molecular Cell Biology and Genetics** could study the microrheology of the protein droplets consisting of PGL-3 protein in four different salt concentrations (75, 115, 150, and 180 mM KCl). As described above (see Experimental design in pages 4-5), they first brought both trapped beads into adhesive contact with the droplet. Next, they applied directional force on one bead, bidirectionally moving it in the x-axis to deform the PGL-3 droplet. Since the force needed to move the trap changes with altered viscosity, Jawerth et al. could measure forces in different salt concentrations to assess the changes.

The team found that both the viscosity and surface tension of the protein droplet decreased with increasing salt concentrations (1 to 0.1 Pa s and 5 to 1 μN/m, respectively: **Figure 8**). The results indicate that electrostatic interactions influence the material properties of these protein droplets, altering the interactions in a concentration-dependent manner.

Application ideas

Staying ahead of the curve

- Observe the formation of droplets using tools like Fluorescence Correlation Spectroscopy/FRAP at different concentrations.
- Study fusion or splitting of droplets as a function of size concentrations, buffer conditions, or protein components.
- Detect inhomogeneities within a droplet by moving the integrated trapped bead in all orientations.
- Create complex systems associated with cellular structures, such as chromosomes, by sequentially adding complexity.
- Study droplet transport in biological systems, such as synapses.



Conclusion

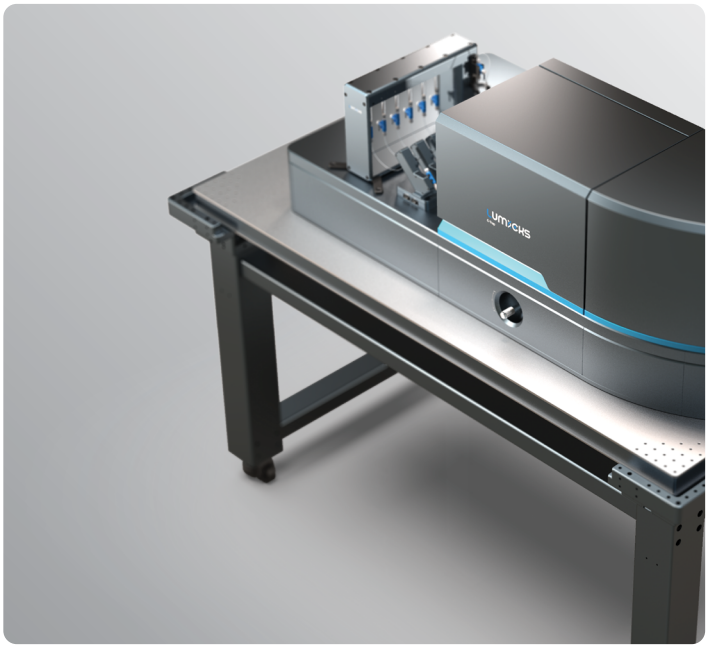
The field of phase separation is emerging and will require increasingly detailed and mechanistic data to understand the processes entirely.

Detailed analyses of liquid–liquid phase separation properties will not only aid our fundamental understanding of natural cellular processes but will also promote the future development of drug therapies. The latter includes the development of inhibitors targeting the transition between liquid compartments and pathologic aggregates, commonly found in neurodegenerative diseases.

The solution: The C-Trap® Dymo

The C-Trap Dymo seamlessly combines highly dynamic and sensitive measurements with a variety of microscopy techniques. You can choose between widefield, confocal, stimulated emission depletion (STED), and label-free microscopy to visualize any interaction between biomolecules (from RNA-binding proteins to the formation of ribonucleoproteins). On top of that, the system supports up to three channels of fluorescent excitation, which allows you to identify different proteins and their co-localization in a protein droplet.

The C-Trap Dymo is a powerful tool that complements and expands the phase separation field allowing you to study physiologically relevant conditions in real time.



Acknowledgements

You can find the experiments presented in this application note in the following publications:

- Kaur et al. 2019, published in Biomolecules.
- Alshareedah et al. 2019, published in the Journal of the American Chemical Society.
- Gui et al. 2019, published in Nature Communications.
- Jawerth et al. 2019, published in Physical Review Letters.

We want to express extra gratitude to Dr. Priya Banerjee and his lab for providing us with additional material for this application note.

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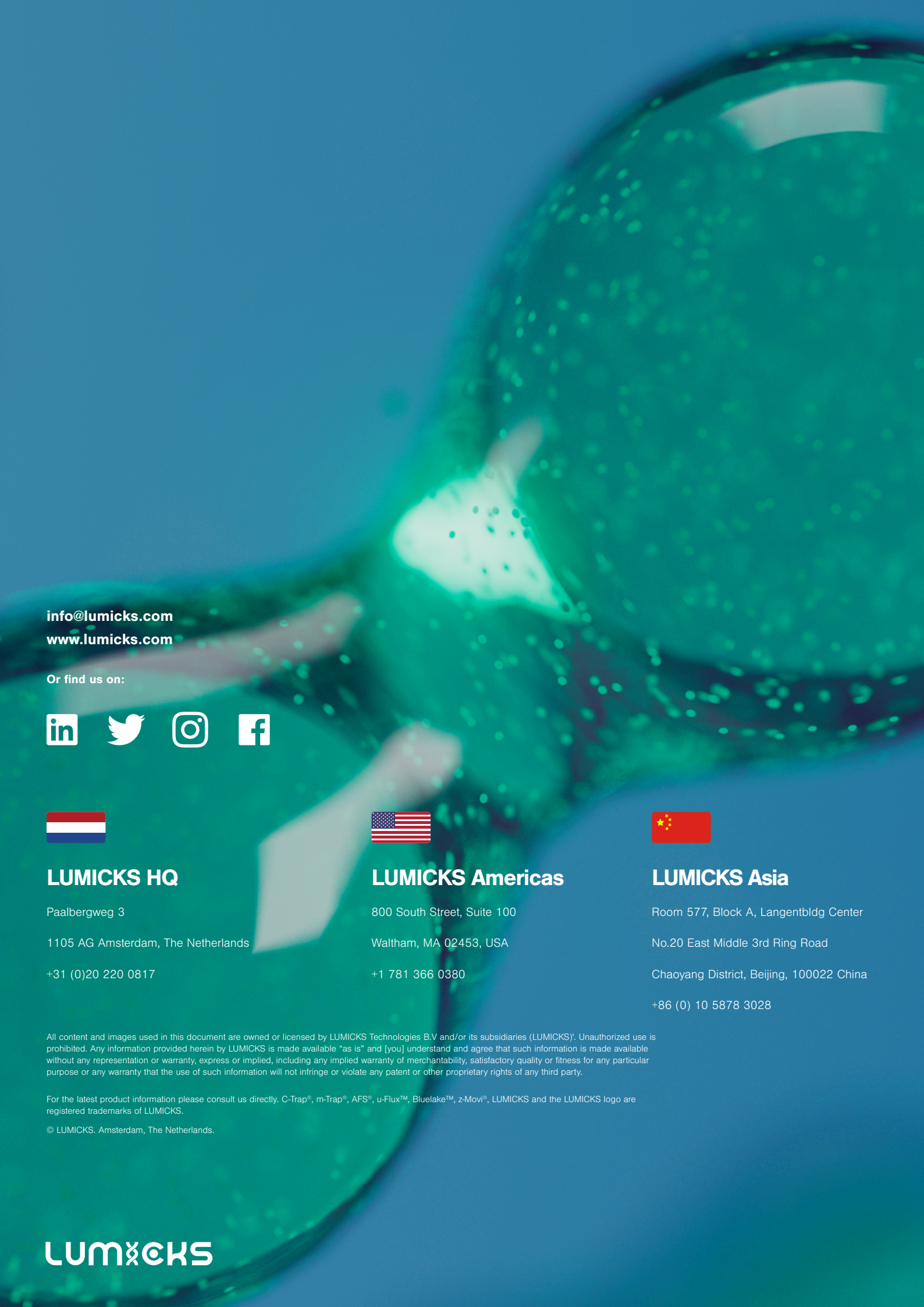

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