Supplementary Material (ESI) for Lab on a Chip

Mapping and Manipulating Temperature-Concentration Phase Diagrams Using Microfluidics

Šeila Selimović,[a] Frédéric Gobeaux[b] and Seth Fraden*[a]

Supporting information

A. Device structure and experimental setup

Device structure:

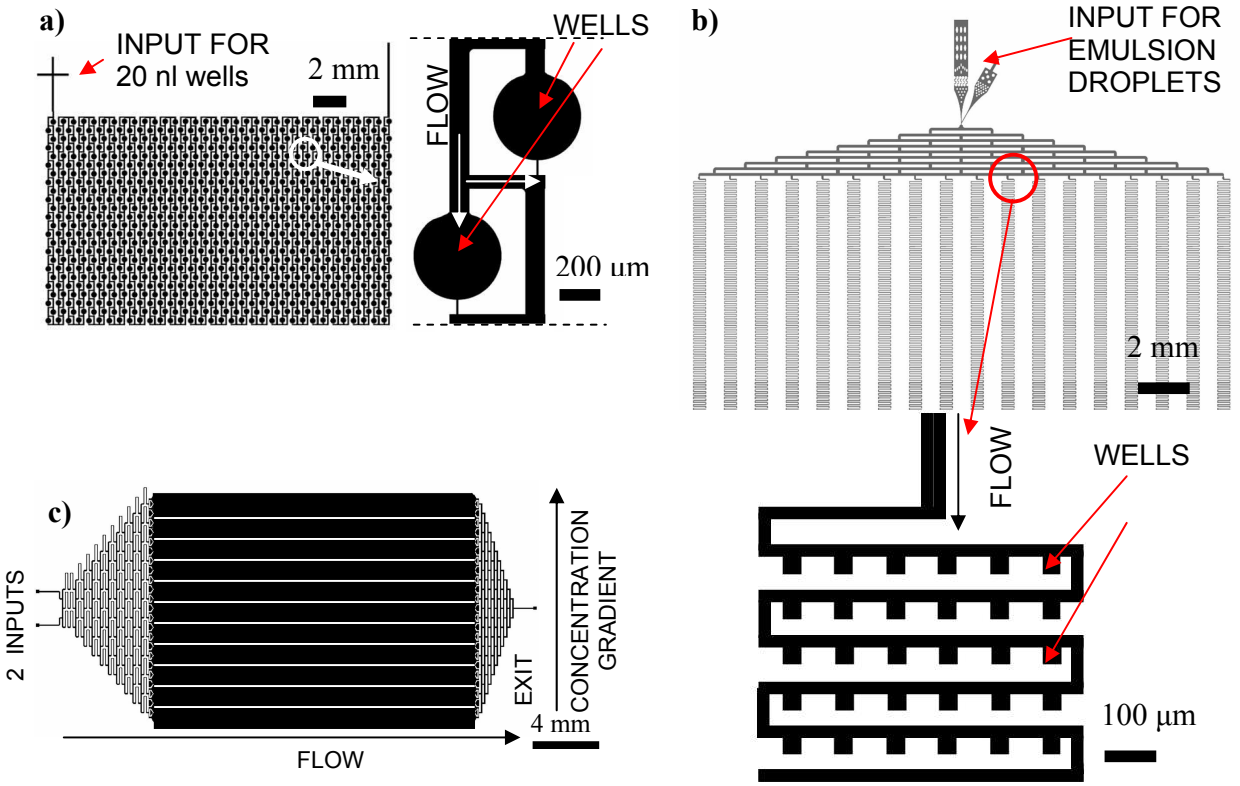

Figure S1. (a) Drawing of the "20 nl PhaseChip": low-resolution drawing of the storage layer and high-resolution image of a unit cell that contains two storage wells. The well diameter is 600 µm. **(b)** Drawing of the "Emulsion PhaseChip" for storing surfactant stabilized drops: low-resolution drawing of the storage layer and high-resolution image of thirty wells. Height and width of the flow channels are 50 µm, while the wells are deeper with dimensions of 50 µm x 60 µm x 70 µm (length x width x height). **(c)** Schematic of the reservoir layer used for both PhaseChip variants. Two inlets for two different salt solutions are shown on the left. As the solutions flow to the right, they mix in the triangular tree-like region on the left until they reach the main reservoir channels. Once the solutions have passed this region, they exit on the right.

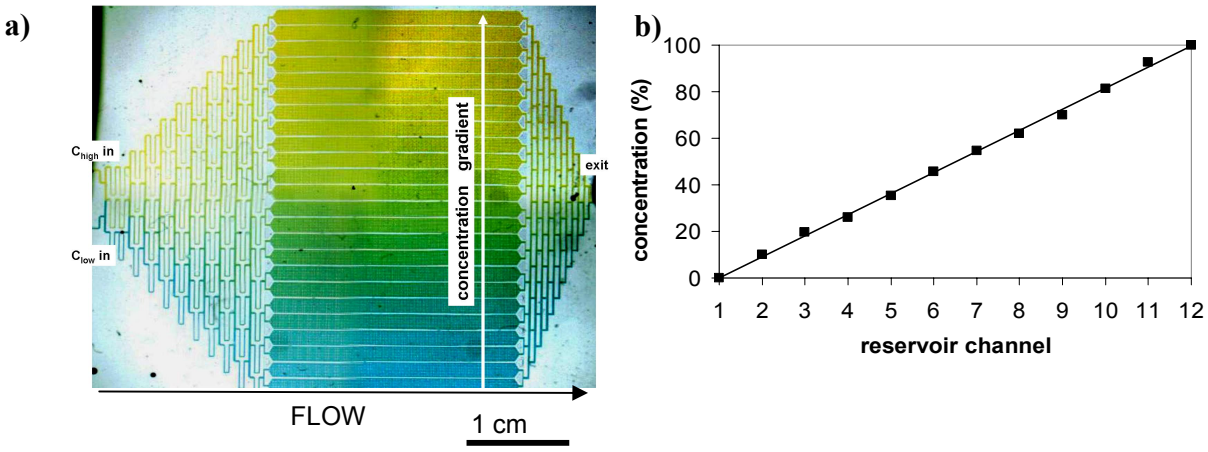

Figure S2. (a) Linear concentration gradient in the reservoir channels visualized with yellow and blue dyes (highly concentrated NaCl solution and pure water, respectively). The direction of the gradient is perpendicular to the flow direction. **(b)** Linear concentration profile of fluorescent dye in aqueous streams across twelve reservoir channels (■). The solid line is a linear fit to the data. The two aqueous solutions used in this experiment are pure water and water carrying Alexa 488 fluorescent dye. Photographs of the mixing streams were analyzed using ImageJ software (http://rsbweb.nih.gov/ij/).

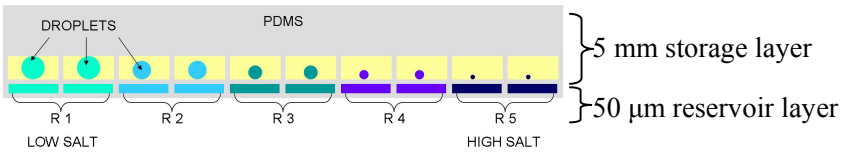

Figure S3. Schematic of the PhaseChip cross-section (simplified). Five, instead of the twelve sets of reservoir channels (R1 - R5) carrying different salt solutions are shown. Each set contains two reservoir channels. While in the "20 nl" storage chip there are two 20nl sized droplets exposed to the same reservoir solution at a particular temperature, in the case of the emulsion device, five droplets are exposed to the same reservoir solution at any given temperature. As the reservoir solution becomes saltier (from left to right), so do the droplets. This is indicated by their reduced size and deeper color (also from left to right).

Drop formation and storage:

The larger droplets of 20 nl are formed via the "store, then create" method we described previously[1], where a long plug of aqueous solution is first stored in the wells and then separated into distinct droplets. The advantages of the "store, then create" method are zero dead volume and the elimination of the need for complex drop formation elements such as nozzles. Typically the 20nl PhaseChip consumes less than 2µl of solution. The carrier fluid in this case is either a mixture of fluorinated oil FC-43 (Acros) and 12% v/v of 1H,1H,2H-2H-Perfluoro-1-Octanol surfactant (Fluka) in protein experiments, or FC-43 alone in polymer experiments. In contrast, the emulsion droplets are formed in a separate PDMS device using a 50 µm wide nozzle.[8] Emulsion droplets are then stored for up to several days in a glass vial, until they are used in an experiment. During this time, no stability or adhesion problems occur, due to the presence of the surfactant. Next, emulsion droplets are injected into the emulsion PhaseChip via polytetrafluoroethylene (PTFE) tubing. Surface tension forces guide the drops into the wells.[8] Here, the carrier fluid is a partially fluorinated oil, 2-trifluoromethyl-3-ethoxydecafluoro hexane (HFE 7500, 3M Novec) with 2% w/w of a proprietary non-ionic triblock surfactant kindly provided by RainDance Technology and similar to the one synthesized by Holtze et al[3]. In any given experiment more than 90% of wells are filled with a single protein or polymer drop and all stored drops have the same initial composition.

a)

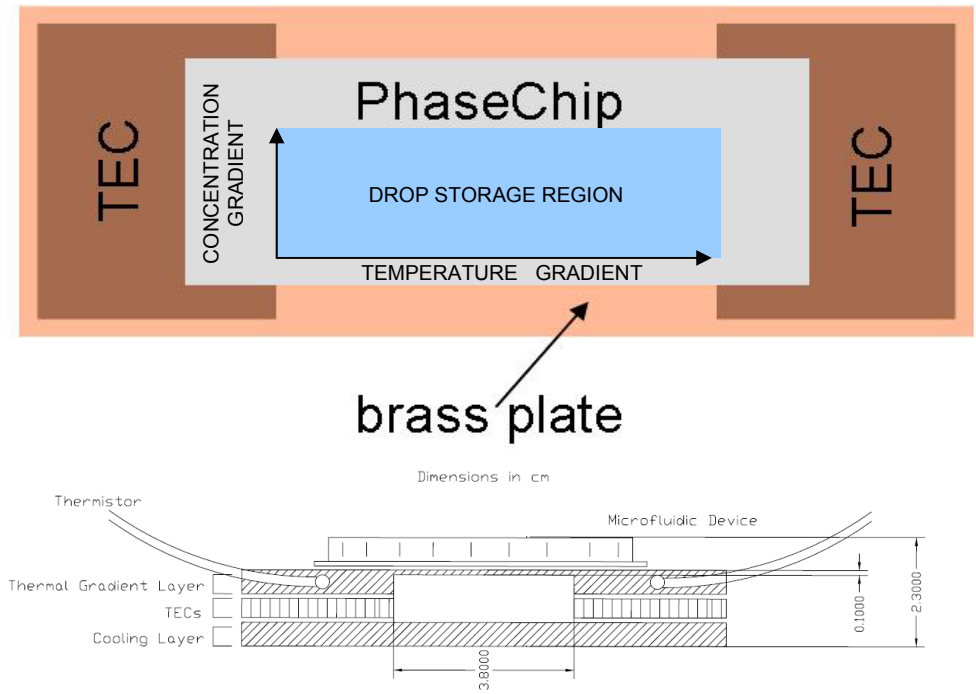

Figure S4. Thermal stage. **(a)** Top view. The microfluidic device is placed on the top of a 1 mm thin brass plate, which in turn is in contact with two thermoelectric coolers (TEC) placed on each end. Water circulates through the brass cooling layer and acts as a source / sink for heat to / from the microfluidic device. Generating a temperature gradient from 2°C to 40°C requires about 20W of power. **(b)** Cross-sectional view.

Measuring solute concentration in drops:

The volume of the drops and shape of the 20 nl wells are such that the drops take on a disk-like shape inside the storage wells, and the height of the drops remains constant at all times. Hence, the change in drop volume only depends on the change in drop area, which we measure using image recognition software (NI Vision Assistant). Protein, polymer and salt concentration in a drop at time t is then obtained from the drop area $A(t)$, the initial area A_0, and the known initial concentration C_0 : $C_0/C(t) = A(t)/A_0$. The emulsion droplets, on the contrary, are spherical throughout the experiment. In this case we measure the drop radius, rather than the drop area, and then calculate the drop volume and deduce the solute concentrations as a function of time.

B. Experimental section:

I) Polymer-salt coexistence: polyethylene glycol (PEG) – ammonium sulfate $(NH_4)_2SO_4$

We determine the concentration-temperature phase diagram of the water soluble polymer PEG 22 kDa in the presence of ammonium sulfate salt ($(NH_4)_2SO_4$**)** in a molar concentration ratio of 1:29.5. Three experiments shown below were done using 20 nl drops to test whether or not the final state depends on the thermodynamic path taken from the initial state. The droplets contain 14.5 mM PEG (Sigma), 427 mM ammonium sulfate (Fisher) and Hannaford blue food dye (5% by volume) at the beginning of each experiment. The polymer solution is prepared and filtered off-chip (0.2 µm syringe filter, Corning, product number 431212), as are the buffers and salt solutions. As the drops shrink the concentrations of PEG and salt grow, but the ratio remains constant at 1:29.5. We record the state of the droplets (single phase or liquid-liquid phase separated) at various temperatures and concentrations. We further conduct two experiments with 65 pl emulsion droplets to determine whether the phase diagram shows a dependence on the drop volume.

a) 20 nl droplets at 14.5 mM PEG (22kDa) and 427 mM ammonium sulfate are stored in the "20 nl PhaseChip" and subjected to a reservoir concentration gradient between 215 mM and 1.33 M ammonium sulfate. The temperature is kept constant at 10°C across the PhaseChip until the droplets have equilibrated with the reservoir solutions underneath. The temperature is then incrementally increased from 10°C to 40°C in steps of 5°C every 2 hours, and finally reduced to 10°C in the same fashion.
b) 20 nl droplets at 14.5 mM PEG (22kDa) and 427 mM ammonium sulfate are stored in the "20 nl PhaseChip" at 8°C, and a temperature gradient between 8°C and 40°C is then applied across the chip. After 15 minutes, a period sufficient for the droplets to equilibrate in temperature, a concentration gradient through the reservoir channels is applied as described in the previous experiment and maintained for an equilibration period of 24 hours.
c) 20 nl droplets containing 9.67 mM PEG (22kDa) and 285 mM ammonium sulfate, again in a ratio of 1:29.5, are stored in the "20 nl PhaseChip" at 20°C, but this time the concentration and temperature gradients (5°C to 38°C) are applied simultaneously. The temperature gradient is chosen such that the difference in temperature between two adjacent drops along the temperature axis is roughly 1°C.
d) 65 pl emulsion stabilized droplets used in this experiment contain 14.5 mM PEG 22 kDa and 427 mM ammonium sulfate (a ratio of 1:29.5), but no dye. We introduce 0.5M and 4M ammonium sulfate solutions into the reservoir in order to regulate the concentration of solutes in the drops. The experiment is conducted at room temperature.

Results:

I a), b), c) A view of the PhaseChip with a clear and a liquid-liquid phase-separated region can be seen in Figure S5a. The red curve serves as a guide to the eye: the blue droplets to the left of it are large and clear, while the drops to the right of the curve are much smaller (because concentrated) and phase separated. Two examples of drops from either side of the red curve are also shown. Concentration-temperature data from all three experiments conducted on the PEG - ammonium sulfate mixture are reported in Figure S5b. The three sets of results match each other, resulting in a phase boundary (solid black line) that divides clear droplets from liquid-liquid phase separated droplets. Thus, it is irrelevant which parameter, concentration or temperature, is adjusted first. A path-independent final state is indicative of an equilibrium phase transition. Once the droplets phase separate into two distinct liquid phases (a polymer-rich and a salt-rich phase), we are unable to determine the concentration of salt and polymer in each phase simply by measuring the volume of the drops. This means that we can determine the phase boundary, but not the composition of the co-existing phases.

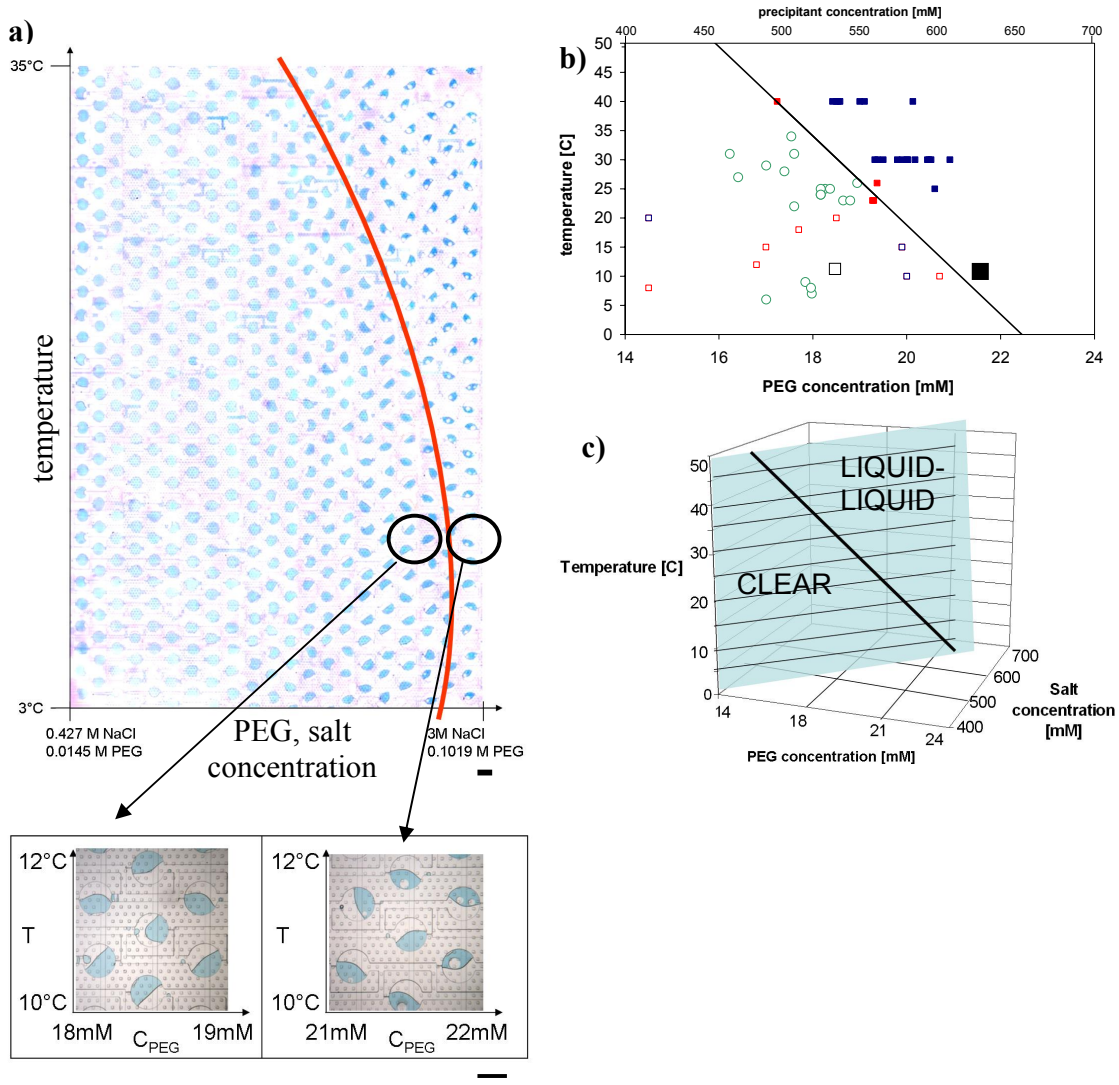

Figure S5. (a) Stitched image of the PhaseChip (630 droplets are shown). The droplets contain a mixture of PEG 22kDa, $(NH_4)_2SO_4$, and blue dye for easier visualization. The axes indicate the applied concentration and temperature range, but not all drops have equilibrated with the applied reservoir concentrations at this time, hence the PEG and salt concentration labels on the concentration axis in this image do not reflect the actual solute concentrations inside the drops. The solid red line serves as visual aid to distinguish the regions of clear drops (left of the curve) and liquid-liquid phase separated drops (right of the curve). The shown area is 1.8 mm x 3.8 mm. Magnified images of clear (left) and phase separated (right) droplets in the PhaseChip are shown below. The labeled concentrations in these images are actual measured solute concentrations inside the drops. Clear drops: C=18 +- 0.05 mM PEG, T:10°-12°C. Phase separated drops: C=22 +- 0.05 mM PEG; T:10°-12°C. Scale bar is 600 µm. **(b)** Phase diagram for PEG 22kDa and ammonium sulfate in the ratio of 1:29.5. Open symbols denote clear drops from all three experiments and filled symbols denote phase separated drops from experiments a) and b). Blue squares denote results from experiment a), red symbols are from experiment b). Green circles denote single phase drops from experiment c). The solid black line is the phase boundary. The magnified images from (a) are shown as large black open and solid squares. Note that the PEG and salt concentrations in the droplets are coupled. **(c)** 3D representation of the phase boundary shown in (c). (Online in color.)

I d) In the emulsion experiment we detect the onset of liquid-liquid phase separation at 18mM $(NH_4)_2SO_4$, a concentration value that is comparable to our measurement made on 20 nl drops at room temperature (19 mM). Figure S6 displays a series of droplets along the concentration gradient. As the two phases separate, the difference in surface tension between them leads to the formation of PEG-rich satellite droplets. (When food dye, which partitions into the salt-rich phase, is used in these experiments, the large droplets turn blue, indicating that the satellite droplets contain a PEG-rich phase.) Such a satellite droplet never detaches completely nor do drops coalesce when they come into contact. We speculate that this is due to the surfactant, which stabilizes the emulsion.

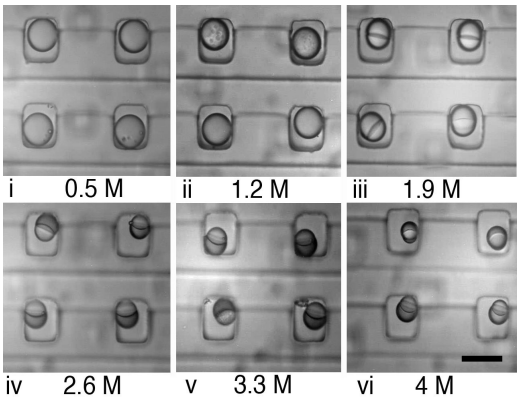

Figure S6. Equilibrium phase separation in emulsion droplets containing a mixture of PEG and $(NH_4)_2SO_4$ is shown at different positions along the concentration gradient in the PhaseChip, at room temperature. The concentration of the corresponding reservoir solution is indicated in each image and the concentration of both PEG and ammonium sulfate increases from (i) to (vi). Scale bar is 60 μm.

II) Liquid-liquid phase separation in protein: Bovine γB crystallin

Crystallins are water-soluble proteins in the eye lens, where they help to increase the refractive index without scattering light, thereby making an optically transparent lens. With advanced age, proteins that are damaged tend to phase separate within each cell. The resulting heterogeneity (cataract) scatters light, rendering the lens opaque.[4,5,6] We use the PhaseChip to demonstrate the ability of bovine γB crystallin to undergo liquid-liquid phase separation in response to changes in temperature and concentration.

The protein sample used in our experiment originally contains 96.5 mg/ml γB crystallin in D_2O ; 50 mM sodium phosphate and 20 mM dithiothreitol at pH 7.1. The choice of D_2O as solvent, together with dithiothreitol, puts the phase separation into a convenient temperature range for our PhaseChip experiments, while at the same time inhibiting a slow oxidation of γB crystallin that can, with time, bias the determination of the phase boundary. The reservoir solutions are pure water and 400 mM sodium phosphate. The temperature gradient originally spans 1°C to 22°C, and is later reduced to a range between 12°C and 18°C.

Results:

At the initial concentration, the protein phase separates at 6.5°C. At higher concentrations the boundary occurs at progressively higher temperatures. Figure S7a shows an image of the PhaseChip 20 hours after the start of the experiment, with temperature (in °C) and concentration (mg/ml) ranges indicated along the arrows. At this time the droplets have not yet equilibrated in concentration with the reservoir solutions. The dashed line demarcates the phase boundary. At temperatures above the boundary the drops appear clear, indicating the presence of a single phase solution. At temperatures below the boundary the protein droplets begin to undergo liquid-liquid phase separation and thus appear dark. Under high magnification one observes tiny globules of a gel. The interpretation is that the solution starts to bulk phase separate into coexisting liquid-liquid phases, but is

kinetically arrested on the high concentration end by the intervention of gelation[2,7]. Magnified images of the PhaseChip at two different conditions are included in Figure S7b, to make the distinction explicit between clear and phase-separated drops.

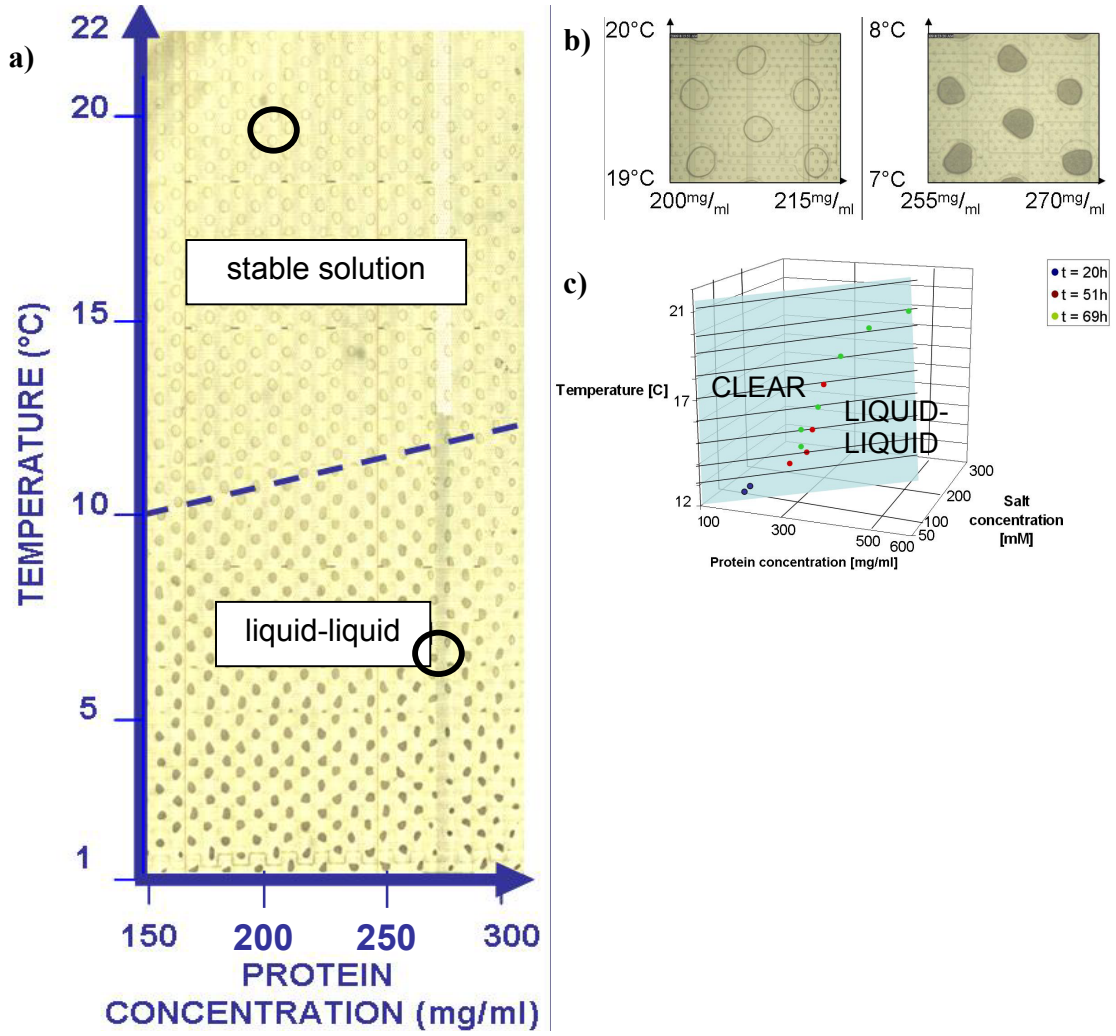

Figure S7. **(a)** Photograph of the PhaseChip 20 hours after the start of the experiment. The dashed line demarcates the phase boundary between single phase and liquid-liquid phase separated regions. **(b)** Magnified images of two sets of protein droplets, marked with circles in a). Left: clear droplets (20°C, 200mg/ml γB crystalllin). Right: turbid droplets (8°C, 270mg/ml γB crystalllin). **(c)** Phase boundary of γB crystalllin, assembled from data accessible at different times throughout the experiment.

After the solute concentration in the droplets has further increased, we reduce the width of the temperature gradient to a range between 12°C and 18°C across the full length of the drop storage region. Then the temperature difference between two adjacent drops along the temperature gradient is only 0.2°C, allowing us to determine more precisely the position of the phase boundary. The resulting images of the PhaseChip can be seen in Figure S8. We note that although the range of measured concentrations and imposed temperatures on the PhaseChip changes throughout the experiment, and thus the phase boundary moves on-chip, its absolute position remains constant, i.e. it is always measured at the same values of concentration and temperature. In other words, all droplets are at quasi-equilibrium at any given time. Solute concentrations change with a half-life of about 12 hours[8], while the liquid-liquid transition occurs instantly on this time scale. The resulting data is shown in Figure S7c: We measure the temperature, precipitant and protein concentrations at which the phase boundary occurs at different times in

the experiment (at t=20, 51, and 69 hours). As the drops continue to shrink, however, the range of solute concentrations accessible on the PhaseChip changes, such that at each point in time we access a different part of the global phase boundary.

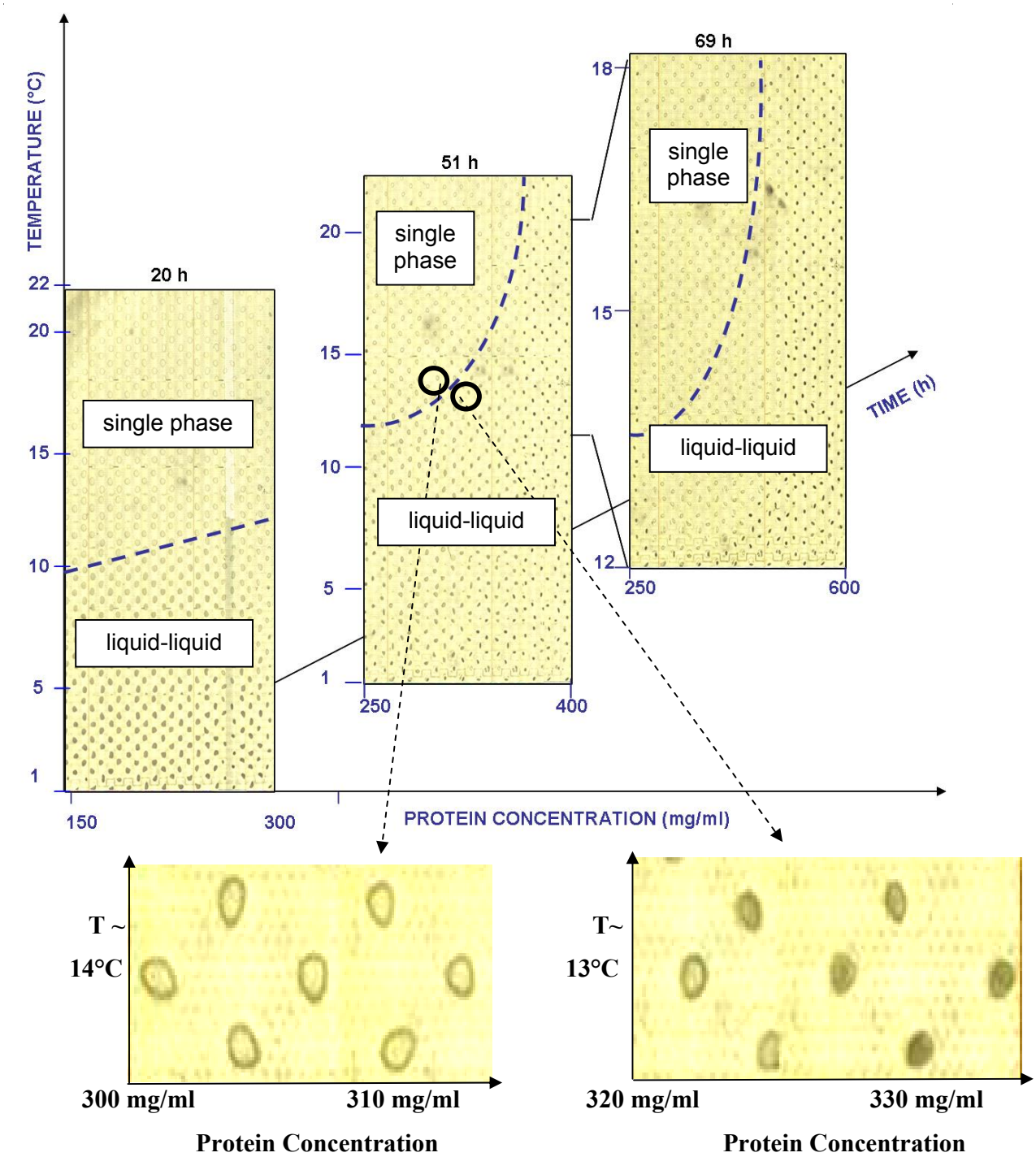

Figure S8. Three images of a single PhaseChip filled with 20 nl droplets of γB crystallin, taken at times t=20, t=51 and t=69 hours after the start of the experiment. The range of protein concentrations (in mg/ml) and temperatures (in °C) accessible in the experiment, as well as the phase boundary (dashed blue line) are indicated individually in each image. (The phase boundary has been determined after careful analysis of high resolution images and is more difficult to recognize in the above views of the full PhaseChip.) Note that although the phase boundary shifts in space throughout the experiment, its absolute position in terms of concentration and temperature values remains constant.

III) Studying phase separation and salvage pathways in a Lysozyme - PEG mixture using emulsion droplets

The goal of this experiment is to evaluate the reversibility of phase changes in the protein sample and probe different salvage pathways[2,8]. We use a mixture of 16.5 mg/ml Lysozyme (Sigma, product number L7651), 2.5% w/v NaCl (Fisher) and 6.25% w/v PEG 8kDa (Sigma), dissolved in 0.05 M NaAc buffer at pH 4.8. The concentration gradient in the reservoir first spans a range from pure water (0 M NaCl) to 1.4 M NaCl, then later a range between 2 M NaCl and pure water, such that in the second step the direction of the concentration gradient is reversed. The temperature is initially set to 20°C in order to prevent premature phase separation and is constant across the chip. After the droplets have equilibrated with the reservoir (ten hours after the start of the experiment), the temperature is briefly decreased to 0°C, then increased to 25°C in 2°C steps. Then, we invert the concentration gradient by switching the two reservoir solution inputs as described above, and after a second equilibration time at 25°C the temperature is again reduced to 0°C (Figure S9a).

Results:

The temperature and concentration profiles in this experiment are shown in Figure S9a: We establish a concentration and temperature gradient, observe the phase behavior, then invert both gradients to explore the reversibility of the phase changes in the droplets. The PhaseChip allows us to conduct many experiments in the concentration - temperature space simultaneously, and the approach taken here lets us explore the phase diagram of the Lysozyme-PEG mixture until we observe crystals in some of the drops, indicating the kinetic pathways and concentration-temperature conditions appropriate for protein crystallization.

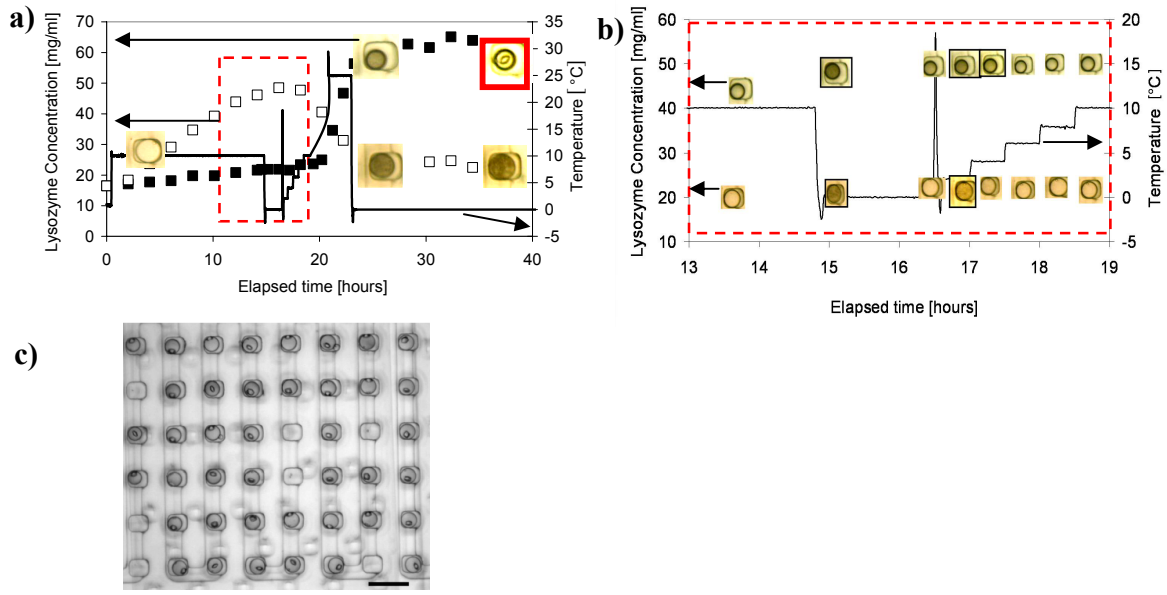

Figure S9. **(a)** Temperature - concentration profiles of two populations of droplets (open and closed icons), which are subjected to two opposite concentration gradients (from 1 to 21 hours and 21 to 40 hours). The temperature profile is the same for all droplets (full black line), but their concentrations vary relative to each other and as a function of time. The inset photographs show typical drops for the concentration corresponding to their position in the figure. The temperature is indicated by the value of the black line at the corresponding time. For example, the upper right most drop (containing a crystal) has a concentration of 58 mg/ml and a temperature of 0°C. Different phases in the droplets are observed: clear (t=0), dark (gel phase), clear drop with a tetragonal Lysozyme crystal. **(b)** This diagram is a magnified version of the dashed red rectangle shown in (a). The position of the photographs indicates their concentration at a given time. The corresponding temperature is given by the solid line. At temperatures below 4°C the droplets become darker indicating the formation of a liquid-liquid phase separation or a gel. (Images containing gels are surrounded by a dark border.) **(c)** An area of the PhaseChip filled with emulsion droplets. The kinetic path and concentration - temperature data for this set of drops is shown in Figure a) and represented by the drop containing a crystal (the image surrounded by a red frame). Each drop contains exactly one tetragonal Lysozyme crystal. Scale bar: 120 µm. (Online in color.)

All droplets are initially clear. As the solute concentration increases to 20 - 50 mg/ml Lysozyme, the droplets become cloudy and develop a gel-like phase below 5°C,[6] but remain clear at higher temperatures. At higher concentrations (60 - 70 mg/ml) and 5°C the droplets are still cloudy, but when the temperature is further reduced (to 0°C), the cloudy phase clears up until crystals are observed (see Figure S9a and Supplementary Movie SM1). A section of the PhaseChip with 40 such emulsion droplets is shown in Figure S9c. This image indicates that a protein supersaturation level necessary for crystal nucleation has been reached in all 40 droplets, hence the chip offers results that are statistically meaningful. Drops that are kept at a low temperature (0°C), but also low concentration (25 mg/ml Lysozyme), however, exhibit different behavior: here the gel phase does not clear up, indicating that the protein is not concentrated enough to facilitate crystal nucleation and growth. These observations suggest that the phase transition from single phase to gel is reversible, but that the protein solution is not in equilibrium due to the crystal nucleation barrier. We further note that a droplet containing cloudy phase rather than a protein crystal does not have to be abandoned in the crystallization process, but can be salvaged by controlling the concentration and temperature of the protein on the PhaseChip.

C. Supplementary Movie SM1

This movie illustrates the final sequence of experiment III (shown in Figure S9a and S9c). We observe 48 wells with a filling ratio of 80%. The drops appear cloudy at first, then, as the temperature is increased, the drops become clear and Lysozyme crystals appear. The time span showed in the movie is t = 28 hours to t = 40 hours.

D. PhaseChip design (PhaseChipMask.pdf)

1 H. Boukellal, S. Selimovic, Y. Jia, G. Cristobal, and S. Fraden, *Lab Chip*, 2009, **9**, 331-338.
2 W.C.K.Poon, *J. Phys. Condens. Mat*. 2002, **14**, R859–R880.
3 C. Holtze, A.C. Rowat, J.J. Agresti, J.B. Hutchison, F.E. Angilè, C.H.J. Schmitz, S. Köster, H.D. Katherine J. Humphry, R. A. Scanga, J.S. Johnson, D. Pisignano, D. A. Weitz, *Lab Chip,* 2008, **8**, 1632-1639.
4 G.M. Thurston, *Proc. Nat. Acad. Sci. USA*, 2007, **104**, 18877-18878.
5 M.L. Broide, C.R. Berland, J. Pande, O.O. Ogun, G.B. Benedek, *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 5660-5664.
6 G.M. Thurston, *Journal of Chemical Physics*, 2006, **124**, 134909.
7 M. Muschol and F. Rosenberger. *Journal of Chemical Physics*, 1997, **107**, 1953-1962.
8 J.U. Shim, G. Cristobal, D.R. Link, T. Thorsen, Y.W. Jia, K. Piattelli, and S. Fraden, *Journal of the American Chemical Society*, 2007, **129**, 8825-8835.