

**Printing life-inspired sub-cellular scale compartments with autonomous molecularly-crowded confinement**

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A simple, rapid and highly controlled platform to prepare life-inspired sub-cellular scale compartments by inkjet printing has been developed. These compartments consist of fL-scale aqueous droplets (few  $\mu\text{m}$  in diameter) incorporating biologically relevant molecular entities with programmed composition and concentration. These droplets were ink-jetted in nL mineral oil drop arrays allowing for lab-on-chip studies by Fluorescence Microscopy and Fluorescence Life Time Imaging. Once formed, fL-droplets are stable for several hours, thus giving the possibility of readily analyse molecular reactions and their kinetics and to verify molecular behaviour and intermolecular interactions. Here, we exploit this platform to unravel the behaviour of different molecular probes and biomolecular systems (DNA hairpins, enzymatic cascades, protein-ligand couples) within the compartments. The fL-scale size induces the formation of molecularly crowded confined shell structures (hundreds of nm in thickness) at the droplet surface allowing discovering specific features (e.g. heterogeneity, responsivity to molecular triggers) that are mediated by the intermolecular interactions in these peculiar environments. The presented results indicate the possibility of using this platform for designing nature-inspired confined reactors allowing deepening our understanding of molecular confinement effects in living subcellular compartments.

## 1. Introduction

Engineered aqueous compartments with life-like features have stimulated a great interest in the recent years since their application as artificial cells and organelles.<sup>[1]</sup> The interest for these systems mainly derives from their development as medicine transport systems.<sup>[2]</sup> Also, artificial and hybrid cells may constitute functional systems to be incorporated in living cells as artificial organelles modulating their functions and processes.<sup>[3]</sup> For example, artificial organelles with light-harvesting capabilities allow for a sustainable ATP energy source,<sup>[4]</sup> for removing antibiotic in surface waters<sup>[5]</sup> and for directing intravesicular reactions.<sup>[6]</sup> On this respect, it is crucial to recapitulate life-like activities in cellular-mimicking environments such as those compartments enclosing selected sets of biomolecular machineries.<sup>[6],[7]</sup>

As a fundamental feature, eukaryotic living cells, which typically enclose pL-scale volumes (about 10  $\mu\text{m}$  in diameter), localize biochemical machineries into smaller compartments driven by liquid/liquid separation or in membrane-defined organelles. In particular, the more complex activities (transcription, energy production, m-RNA processing, etc.) are orchestrated inside heterogeneous fL-scale compartments (nuclei, mitochondria, P-bodies, etc.) that control the spatiotemporal assembly of biochemical processes under highly regulated conditions.<sup>[8]</sup> Their diameter is in the range 0.3-5  $\mu\text{m}$ , depending on the enclosing biomolecular systems and their specific features (concentration, degree of molecular self-assembly, solubility, presence of a membrane).<sup>[8]</sup> On the other hand, more simple activities like transport, signalling and storage are carried out by even smaller compartments, as the synaptic vesicles, which may reach nanoscopic diameters with volumes in the aL- scale and nanometer size.<sup>[9]</sup>

The above different size regimes from the pL to the aL scale are expected to present distinct physico-chemical features also in connection to their different surface/volume ratios.

Focussing on the subcellular systems with more complex activities, different biomacromolecules (DNA, proteins, enzymes) are highly crowded occupying a large part of the compartment volume.<sup>[10]</sup> Crowding may induce volume exclusion, i.e. limited accessible space and available water molecules for biomolecules.<sup>[10]</sup> Moreover, crowding is often associated to confinement effects, as evolution has selected the spatial localization of fundamental processes by spontaneous phase separation processes.<sup>[11]</sup> On this respect, examples include DNA transcription process in bacterial cells<sup>[12]</sup> or DNA packing in eukaryotic nuclear environments,<sup>[13]</sup> or mRNA processing enzymes in P-bodies.<sup>[14]</sup>

Crowding and confinement have been not reproduced synergically in micrometric fL-scale sub-cellular artificial compartments and the correlation between their peculiar surface/volume ratio and the resulting molecular spatial organization and activity needs to be understood. Recent studies show crowding effects in larger pL-scale droplets (about 25  $\mu\text{m}$  in diameter) giving heterogeneous gene expression environments<sup>[15]</sup>. On the other hand, molecular confinement has been already observed to increase the reaction rate and stereo selectivity of self-assembling nanoflasks,<sup>[16]</sup> the ribozyme cleavage rate in aqueous compartments<sup>[17]</sup> and the spontaneous remodelling of actin networks in ring-shaped bundles.<sup>[18]</sup>

For these reasons, it is urgent to provide a strategy to combine crowding and confinement of biomolecular systems in artificial subcellular like fL-scale compartments. Different approaches can produce compartments at this scale.<sup>[19–24]</sup> Direct water-in-oil emulsification permits to obtain water droplets dispersed in oil typically varying in a wide range size (from micro- to nano-scale).<sup>[19]</sup> Also, DNA nanotechnology provides assembled structures mimicking cellular modules, but it suffers complexity and high costs.<sup>[20]</sup> Microfluidics approaches enable the preparation of monodisperse fL-scale droplets under high shear stresses, ultimately modifying the distribution of the surfactants needed to stabilize the oil–water interface. In turn, this can lead to the nonspecific adsorption of biomolecules at the oil–water interface which can potentially affect the biological activity.<sup>[21]</sup> Finally, bio printing

approaches can produce fL-droplets with few micron nozzles in pin-printing,<sup>[22]</sup> inkjet-printing<sup>[23]</sup> or electric field dispensing,<sup>[24]</sup> but these can lead to nozzle clogging or require electrolyte media which again may alter the biomolecular activity.

This paper reports on the realization of a novel platform allowing for the direct printing of life-inspired artificial compartments with well-defined composition and concentration as well as tight dimensional distribution within size scales from the nL to the fL allowing showing the transition effects in the molecular and intermolecular features of incorporated molecular and biomolecular systems. In particular, these compartments are injected in mineral oil drops arrayed on glass substrates by inkjet printing, whereas the molecular structures and activity of the incorporated systems are investigated by advanced fluorescence microscopy and Fluorescence Life Time Imaging (FLIM). Interestingly, just in the few  $\mu\text{m}$  regime novel features emerge by autonomous molecularly-crowded confinement effects.

## 2. Results and Discussion

### 2.1. Printing highly controlled sub-cellular scale aqueous compartments

Nanoliter and picoliter-scale homogenous aqueous droplets can be successfully injected in mineral oil drops by inkjet printing aqueous inks on glass surfaces.<sup>[25]</sup> Here a novel piezoelectric “spike waveform” (i.e. the voltage vs. time signal given to the piezoelectric actuator) is designed (**Figure 1a**) to meet the singularity of the Navier-Stokes equation in the Eggers’s theoretical model which predicts the reduction of droplet size by minimizing the droplet formation time.<sup>[26]</sup> Accordingly, the droplet downscaling up to 15-40 fL (3-4  $\mu\text{m}$  in diameter) from the inkjet printer pL-scale micro-channels is possible by minimizing the ejection time (**Figure 1b**). In particular, by following the model, it is possible to downscale the droplet diameter below the nozzle size (about 10  $\mu\text{m}$  in diameter), if the ejection time is



kept at values down to 1  $\mu$ s. To this purpose, a novel three-phase waveform signal minimizing the jetting time has been designed. Importantly, also the jetting voltage has to be kept as low as possible (between 12 V and 14 V) to pull the liquid tongue into the nozzle by forming only fL-droplets (**Figure 1c**). On the other hand, at high voltages ( $>15$  V) the liquid tongue coalesces with the fL-droplet (**Figure 1d**) and the droplet regains the standard size of the nozzle. Details on the methodology are reported in the Supporting Information (**Figure S1 and S2**).

The fL-sized aqueous droplets are printed in mineral oil drops (see sections below). In order to avoid molecular leakage into the oil phase, these are spiked under optimized conditions with a mixture of non-ionic surfactants including the di-block copolymer poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (PEG-PPG-PEG) (F108; 0.03% v/v), an efficient amphiphilic stabilizer of oil/water interfaces in emulsion droplets,<sup>[27]</sup> and Tween20 (0.05%), already reported as inhibitor of colloidal aggregation in solution.<sup>[25]</sup>

## 2.2 Size induced molecularly-crowded confined structures

About five thousand aqueous droplets at the fL-scale were injected in about 10 nL mineral oil drops arrayed with a spacing of 1 mm on a glass chip in a  $4 \times 2$  format (see **Figure 2a**). Notably, fL-droplets are not stable in absence of F108, which has been observed to self-assemble at the water/oil interface<sup>[27]</sup> avoiding the above cited molecular diffusion to the oil phase.<sup>[28]</sup> Once injected in mineral oil drops, given the high surface tension gradient between water and oil, aqueous fL-droplets are subjected to Marangoni flows toward the border of the oil drop resulting in an almost-regular droplet pattern (see Supporting Information for details). We initially employed fL-droplets containing fluorescent dyes<sup>[29]</sup> (Alexa647 or Atto655-Biotin) to characterize the drops by confocal fluorescence microscopy. In **Figure 2b**, a representative confocal fluorescence microscopy image at a mineral oil drop edge, that is not

fluorescent, is reported, where a pattern of micron-scale fL-droplets is observed. At the oil drop border, fL-droplets are regularly spaced with a distance of about 15  $\mu\text{m}$ . By means of confocal microscopy it is possible to verify that printed droplets have a spherical shape. Here optical sections of the droplets are acquired at about their equator, showing that the fluorescent signal is confined in annular regions at the droplet surface. The average droplet diameter ( $2r$ ) and annuli thickness ( $A$ ) are  $4.0 \pm 0.6 \mu\text{m}$  and  $0.6 \pm 0.1 \mu\text{m}$ , respectively (**Figure 2b**). In three dimensions this results in confined spherical shells with a volume  $3.4 \pm 0.5$  times smaller than the whole droplets. For simplicity, in the following these structures will be referred as annuli, as their features observed by their optical sections. This geometrical confinement likely results in an up-concentration of molecules in the annuli that is specifically triggered by the droplet size reduction. Indeed, in the case of  $\mu\text{L}$  and  $\text{pL}$  scale drops, dye molecules homogeneously occupy the drop volume (hereafter *homogeneous bulk droplets*; HBDs). In fL-droplets, instead, we observe a remarkable molecular adsorption at the water/oil interface which might be the result of surface energy minimization (see **Figure S3-S4** for details). In **Figure 2c** the fluorescent signal of the above sample in about 50  $\mu\text{L}$  HBDs is presented in comparison with the downscaled fL-droplets with the annular structures.

By employing theoretical models describing the absorption of amphiphilic molecules at the interfaces of immiscible liquids,<sup>[30]</sup> it is possible to reveal that the molecular adsorption layer at the water/oil interface would result in a homogeneous concentration profile of molecules with a thickness ( $L_t$ ) of about  $\sim 500 \text{ nm}$  (see Supporting Information for details). Interestingly, this predicted  $L_t$  value is comparable to that experimentally observed in fL-droplets containing a model dye and the above amphiphilic systems. By considering that the molar ratio between the amphiphiles and the dye is about 1000:1, it is possible to infer that the annulus would consist mainly of F108-Tween20 molecules enclosing the dye molecules.

### 2.3 FLIM analysis of molecular probes in molecularly-crowded confined structures

FLIM has been employed to investigate the physico-chemical properties of fluorescent molecules in the annuli. Analysis of FLIM measurements was performed by the phasor approach,<sup>[31,32]</sup> which gives the graphical view of all the measured fluorescence decays at each pixel of the observed image. Separate clouds of pixels (selected with coloured cursors) in the phasor plot stem for separate fluorescence lifetimes distributions measured at the image pixels.). Long lifetimes are near the origin while short lifetimes are shifted on the circular line toward the bottom right (1 on the x axis). The combinations of two single exponential components lie inside the semicircle and can be described as a linear combination of two mono exponential states.<sup>[32]</sup> It is possible to distinguish different lifetime distributions when they appear well separated in the phasor plot. In the following we will report as  $\tau$  the value of the average lifetime of fluorescence decay measured in pixels selected by the cursor.

The effect of annular confinement was first probed by measuring the  $\tau$  distributions for model dyes (**Figure S5**). The  $\tau$  distributions of the environmental insensitive Alexa 647 dye<sup>[29]</sup> or the model viscosimeter CCVJ 9-(2-Carboxy-2-cyanovinyl) julolidine<sup>[33]</sup> do not significantly change within the annuli (**Figure S5a** and **S5b**) with respect to the one measured in HBDs (see Supporting Information). On the other hand, a significant  $\tau$  reduction in annulus is observed for fluorescein isothiocyanate (FITC), a probe critically sensitive to the pH or the hydrogen-bond character of the environment<sup>[34]</sup> ( $\tau_{\text{ann.}} = 2$  ns;  $\tau_{\text{HBD}} = 4$  ns see **Figure S5c**). This reduction can be likely ascribed to the up-concentration of surfactants<sup>[30]</sup> and/or the water molecules depletion (crowding inducing excluded volume effects) within the annuli.<sup>[35]</sup>

The analysis of fluorescence lifetime distribution of FITC conjugated to the protein Bovine Serum Albumin (BSA) is reported in **Figure 3** together with measurements on the mixture of the two molecules. The phasor plot of **Figure 3a** shows different lifetime distributions marked by three different cursors. The fluorescence lifetime selection maps for HBDs and for the annuli are reported in **Figure 3b** and **Figure 3c**, respectively. The BSA/FITC mixtures (BSA

1.2  $\mu\text{M}$ ; FITC 10  $\mu\text{M}$ ) are shown in the upper panels whereas the BSA-FITC conjugates (BSA-FITC 10  $\mu\text{M}$ ) samples are in the lower. The HBD fluorescence in **Figure 3b** is uniform and with the same lifetime distribution in the whole image both for the BSA-FITC conjugate (magenta cursor) and BSA/FITC mixtures (orange cursor). As expected,  $\tau_{\text{HBD(mix)}}$  is centred at about 4 ns, that is close to the typical  $\tau$  of free FITC.<sup>[35]</sup> On the other hand, the lifetime distribution of BSA-FITC conjugates -  $\tau_{\text{HBDs(conj)}}$ , is found inside the universal circle and it is significantly shorter than  $\tau_{\text{HBD(mix)}}$ , as expected.<sup>[36]</sup> Measurements in the annuli show critically different results, being the lifetime distributions for both the BSA-FITC conjugates -  $\tau_{\text{ann(conj)}}$  and BSA/FITC mixtures -  $\tau_{\text{ann(mix)}}$  superimposed at about 0.5 ns. These two indistinguishable  $\tau$  distributions are selected by the red cursor in the phasor plot (see **Figure 3a**). Accordingly, the pixels in the selection maps are highlighted with the same colour (**Figure 3c**). Interestingly, the  $\tau$  distribution for the BSA-FITC conjugates in the annuli is significantly shorter than in HBDs. Note that the  $\tau_{\text{HBD(conj)}}$  value lies on the straight dashed line (see **Figure 3a**) connecting the  $\tau_{\text{HBD(mix)}}$  and the  $\tau_{\text{ann(mix)}}$ , as an indication that the fluorescence decay is more complex and can be characterised by a double exponential of these two lifetimes.<sup>[31]</sup> These results suggest that the environment experienced by FITC dye mixed in solution with BSA in the annuli is very similar to the one experienced by the same dye covalently bound to BSA. The observed effect can be rationalised by considering that the molecularly-crowded confinement with excluded volume effects can trigger nanometric scale intermolecular proximity.<sup>[37]</sup> Analogous results were found by employing Atto655 labelled biotin interacting with the tetravalent Streptavidin (**Figure S6**). The different lifetime distributions of this model protein-ligand couple in the annuli with respect to HBDs may be ascribed to changes in the Streptavidin conformation and/or to larger quenching effects upon the formation of the Biotin-Streptavidin complex (see Supporting Information for details).

## 2.4 CYP2E1 enzymatic process in the molecularly-crowded confined structures

The data presented in the previous section have shown that molecular crowded confinement is generated in the fL-scale droplets. Here we further investigated this environment by using biochemical reactions characterised by a higher level of complexity, as those typically occurring in cellular environments.

In particular, the assembly of complex metabolic processes in artificial microcompartments is fundamental for the construction of cell-like systems<sup>[1]</sup> and on this respect it is important to optimize the spatial distribution<sup>[38]</sup> and the intermolecular distance<sup>[39]</sup> of enzymatic processes in sub-cellular scale compartments in presence of crowding and confinement. Accordingly, in the crowded cellular environments, metabolic reactions are assembled as spatially organized networks triggered by the interaction between different enzymes in response to chemical gradients of their enzymatic substrates, leading to emerging properties such as substrate channelling<sup>[39]</sup> and chemotaxis.<sup>[40]</sup> Among the enzymatic systems, the Cytochrome P450 2E1 (CYP2E1) is a cytochrome P450 phase I membrane protein whose expression in mitochondria is connected to type 2 diabetes, obesity and ethanol intoxication.<sup>[41]</sup> Importantly, CYP2E1 in the mitochondrial matrix environment shows remarkable differences in the induction of oxidative stress with respect to the cytosol-exposed enzyme in the endoplasmic reticulum.<sup>[41]</sup>

In our experiments, we employ CYP2E1 as a model metabolic enzyme in artificial micrometric compartments in order to mimic a mitochondrial enzymatic process. This process involves the catalysed hydroxylation of 7-(ethoxymethoxy)-2-oxo-2H-1-benzopyran-3-carbonitrile (EOMCC) to a fluorescent product, namely 3-cyano-7-hydroxycoumarin (CHC) together with a regeneration system restoring the CYP2E1 activity through the NADPH+ produced by the reduction of NADP+ with the Glucose 6 phosphate dehydrogenase catalysed oxidation of glucose-6-phosphate (see **Figure S7** for details). Note that this behaves similarly to living systems where the CYP450 metabolic reactions are actively sustained by electron donors to produce active species of the enzymes.<sup>[41]</sup>

Sample droplets with the above described mixture in presence of different concentration of EOMCC (ranging from 5  $\mu\text{M}$  to 40  $\mu\text{M}$ ) have been injected into mineral oil drops. The fL droplets were imaged as a function of time every 20 minutes up to 120 minutes. No significant fluorescence was detected at the beginning, but then the signal progressively grew. In stationary conditions, it is evident that the fluorescence signal, due to the formation of CHC, in line with previously analysed systems, is confined at the edge of the drop and in shells with same spatial features. No further signal increase was observed after 120 minutes. Interestingly, **Figure 4a**, shows that at the lowest EOMCC concentration (5  $\mu\text{M}$ ) the fluorescence signal was discontinuously distributed at the annuli showing CHC-rich separated micron-sized regions (about 2  $\mu\text{m}$  in size), hereafter defined as “spots”. At increasing concentration of EOMCC, the spots in the confined regions grow in number and size until the full annulus is filled at about 20  $\mu\text{M}$ . At difference from measurements in HBD where the fluorescence signal is uniformly distributed, in the fL-droplets confined environment a heterogeneity of the reaction product spatial distribution is observed.

In **Figure 4b**, the phasor plot of the CYP2E1 reaction ( $[\text{EOMCC}] = 20 \mu\text{M}$ ) in the annuli and in HBDs is reported in order to compare the  $\tau$  distributions of CHC fluorescence. It is possible to observe a single distribution centred at about  $\tau_{\text{HBD}}(\text{CHC}) = 3.6 \text{ ns}$  (orange cursor), whereas the  $\tau_{\text{ann}}(\text{CHC})$  distribution is inside the phasor plot at shorter values, and can be considered as the combinations of two single exponential components.<sup>[32]</sup> The corresponding selection maps are reported in **Figure 4c**. Results can be interpreted by considering that CHC-fluorescence reflects differences in its environment. On this respect, hydroxycoumarins, such as CHC, have been observed to experience acid dissociation, leading to both the undissociated and dissociated phenolates with different fluorescence spectra depending on the environmental water content.<sup>[42]</sup> Accordingly, the above water exclusion effects within the annuli would lead to a reduction of  $\tau$  by a significant presence of almost quenched undissociated phenolates which would coexist with the fluorescent dissociated form. The reaction product formation as

a function of the EOMCC concentration is shown for HBDs and for the spots observed in the annuli at the EOMCC concentration of 20  $\mu\text{M}$  in **Figure 4d** and **Figure 4e**, respectively. Fluorescence intensity growth in **Figure 4d** qualitatively takes in account for CHC formation as a function of EOMCC concentration and indicates that the reaction profile in HBDs is in good agreement with experiments in larger bulk volumes.<sup>[43]</sup> As shown in **Figure 4f** where the average fluorescence measured in the spots is reported as function of the EOMCC concentration, an analogous behaviour is observed indicating that such a complex reaction is still occurring in the annuli of fL droplets.

Notably, heterogeneous distribution of products in enzymatic reactions, highlighted in these experiments, has previously been observed in other enzymatic systems. Recently, Zhao et al. observed spots at similar sizes in microfluidic-based platforms, due to glycolysis cascade enzyme chemotactic assemblies induced by their specific substrates in crowded conditions.<sup>[44]</sup> In our case, the localization of CHC in spots can be the result of crowding in environments with limited water content, i.e. a condition where protein-protein interactions have been observed triggering phase separation in intracellular environments.<sup>[11]</sup> On the other side, at high EOMCC concentrations, the more uniform CHC signal from the annuli would be consistent with the lateral growth of the CHC-rich zones as well as with a lower probability to generate chemotactic-driven enzymatic assembly.<sup>[44]</sup> The CYP2E1-catalyzed reaction in highly concentrated regions (annuli and/or spots) may be interestingly related to its different reactivity in mitochondria and in endoplasmic reticula.<sup>[41]</sup> Indeed, the different reactivity takes account not only of the enzyme-substrate affinity (in mitochondria CYP2E1 has been observed with a more unfolded conformation and a markedly reduced  $\alpha$ -helical contents compared with the cytosolic CYPs<sup>[41]</sup>) but more specifically of the localization of the whole enzymatic processes involving the enzyme regeneration system and/or the CYP2E1-mediated production of reactive oxygen species finally producing more toxic effects in mitochondria than in the cytosol.<sup>[41]</sup>

## 2.5 DNA hairpins in the molecularly-crowded confined structures

DNA hairpins are reactive DNA structures formed by intra-strand base pairing of a sequence containing two self-complementary portions.<sup>[45]</sup> They are used here as model systems to evaluate the folding/unfolding behaviour of biomolecules in artificial subcellular-like environment.<sup>[46],[47]</sup> **Figure 5a** shows the DNA hairpin with a fluorophore (Alexa555) at 5' and a Black Hole Quencher (BHQ-2) at 3' allowing for Förster resonance energy transfer (FRET). Hybridization of the DNA hairpin with a DNA sequence complementary to the loop portion (input) converts the hairpin into a double-helical segment with Alexa 555 away from the quencher, thus allowing for fluorescence emission (ON-state). **Figure 5b** reports the phasor plot with the  $\tau$  distributions of Alexa 555 bound to the DNA hairpin (10 nM) in the absence (OFF-state) and in the presence (ON-state) of the DNA input (500 nM) for both HBDs and the annuli in fL-droplets. Molecules in the annuli are about 3.4-fold more concentrated than in HBDs. Control experiments carried out in HBDs at about 3.4-fold higher concentration, i.e. a condition where all molecules and buffer concentrations are similar to that in the annuli, exclude up-concentration effects since no differences in the  $\tau$  distribution are observed. The Alexa 555  $\tau$  in the OFF-state in HBDs is not measurable, while in the annuli (magenta cursor)  $\tau_{\text{ann}}(\text{OFF})$  is about 0.2 ns. In the same environment, the DNA ON-state shows in the annuli (red cursors)  $\tau_{\text{ann}}(\text{ON}) = 0.9$  ns to be compared to that in HBDs (orange cursor)  $\tau_{\text{HBD}}(\text{ON}) = 0.6$  ns. The corresponding selection maps for all the analysed samples are reported in **Figure 5c**.

In order to monitor the DNA hairpin OFF to ON switch, droplets containing mixtures of DNA hairpin and DNA input were printed and the fluorescence signal was collected after 30 minutes. Representative images of fL droplets containing Alexa 555-DNA hairpin showing a fluorescence growth with the printed concentration of the DNA input (from 1 nM to 1  $\mu$ M)



are reported in **Figure 5d**, the fluorescence signal being homogeneously localized in the annuli with thickness of  $580 \pm 70$  nm. The fluorescence intensity of DNA hairpins as a function of the DNA input amount is reported for HBDs and for the annuli in **Figure 5e** and **Figure 5f**, respectively. As expected, DNA hairpin in HBDs shows an insignificant fluorescence signal due to the Alexa555 quenching by BHQ-2 (the measured fluorescence intensities are comparable to the background noise). On the other hand, as reported in **Figure 5d**, DNA hairpin in the annuli shows a non-zero fluorescence in absence of input. DNA hairpin signal is completely quenched in 3.4-fold up-concentrated HBDs, so that we can exclude that this effect is due to up-concentration and it might be more likely ascribed to the interaction between the molecular systems with the confined environment and/or to a slight difference in the initial DNA hairpin conformation.<sup>[47]</sup> Notwithstanding this difference, the fluorescence signal as a function of the input concentration shows in the annuli of fL-droplets again a sigmoidal shape indicating an efficient ON-OFF switching.

### 3. Conclusion

This paper reports on the development of a new versatile lab-on-chip platform to prepare and characterize life-inspired subcellular compartment very well defined in dimension, composition and concentration by advanced printing and fluorescence techniques.

From the fundamental point of view, we demonstrated the onset of different new features by precisely downscaling at the fL-volume or few  $\mu\text{m}$  size regime, i.e. the same dimension of sub-cellular complex compartments. In contrast to larger droplets, this size regime results in the spontaneous confinement of hundreds of nm thin shell molecular structures at crowded liquid/liquid interfaces stabilized by amphiphilic molecules. These structures enclose crowded assemblies of selected molecular or biomolecular systems (**Figure 6a**). In effect, nature employs analogous phase separation mechanisms to include precious sets of functional biomolecules within membrane bound organelles (e.g. mitochondria, nuclei) and membrane-

less systems (P-bodies, Cajal bodies, etc.).<sup>[11]</sup> Thus, in addition to the intermolecular interaction, for these systems their complex function within the cellular economy is likely related to their size regime allowing for both enough volume space and a peculiar surface/volume ratio driving phase separation phenomena.<sup>[8]</sup> Accordingly, other sub-cellular compartments typically experiencing more simple functions (e.g. transport) may take smaller (nanoscopic) sizes not showing the above structural features.<sup>[8]</sup>

Emerging properties of the here studied confined systems include up-concentration, molecular proximity and decreased water content. Our studies reveal that the crowded confinement triggers an unprecedented heterogeneity in the spatial organization of reaction products generated by mitochondrial enzymatic reactions (**Figure 6b**). This highlights the importance of the spatial distribution and the intermolecular distances in fL-scale sub-cellular compartments. Notwithstanding confinement might induce subtle conformational changes to biomolecules (i.e. proteins and DNA hairpins), these maintaining their functional features and responsivity to molecular triggers (**Figure 6c**).

We believe that the above findings would result in different technological and fundamental perspectives. At first, the above lab-on-chip platform provides an important tool for discovering the roles of confinement and crowding in the functionality of cellular and sub-cellular compartments. Also, this platform may provide an alternative approach to prepare programmed protocells as well as very well defined artificial smart organelles to be incorporated in living cells to complement or affect the cellular processes.<sup>[3]</sup> Finally, it will be possible to study the correlation between the structure, the properties and the function of molecular assemblies in very well defined sub-cellular compartments under very well controlled physiological and pathologic conditions.<sup>[11]</sup>

#### 4. Experimental Section

*Chemicals and Solution Preparation.* Mineral oil (light oil-neat, Sigma Aldrich), 1H,1H,2H,2H-perfluorooctyltriethoxysilane (Sigma-Aldrich), Tween20 (polyoxyethylene (20) sorbitan monolaurate, Sigma Aldrich), Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) – F108, Alexa 647 (Alexa Fluor® 647 carboxylic acid, succinimidyl ester, ThermoFisher Scientific), Fluorescein Sodium (FITC, suitable for fluorescence, Sigma Aldrich), Bovine Serum Albumin (BSA, Sigma-aldrich, lyophilized powder, ≥96.0%), Albumin-Fluorescein isothiocyanate conjugate bovine (BSA-FITC, Sigma-aldrich), 9-(2-Carboxy-2-cyanovinyl)julolidine BioReagent (CCVJ) (Sigma-aldrich suitable for fluorescence, ≥97.0% HPLC), Atto655 biotin (Bio-Reagent, suitable for fluorescence, Sigma Aldrich), and Streptavidin from *Streptomyces avidinii* (lyophilized from 10 mM potassium phosphate, Sigma Aldrich) were purchased and used as received. With the exception of DNA hairpin and CYP2E1 assay, a buffer solution containing 100 mM phosphate (pH 7.4), 0.05% v/v Tween-20 and 0.03% w/v F108 was employed for all the experiments. In the case of DNA hairpin solutions, NaCl at the final concentration of 50 mM was added. All aqueous solutions were prepared in ultrapure Millipore water (Direct Q-UV filtration system, 18.2 MΩ cm). The pH was controlled by a pH meter (pH 700, Eutech Instruments).

*DNA sequences.* All oligonucleotides were synthesized, labeled, and purified (HPLC and reverse phase) by IBA GmbH (Göttingen, Germany). Oligonucleotides were dissolved in PBS buffer at a concentration of 100 μM, confirmed using Tecan Infinite M200pro (Männedorf, Switzerland) through NanoQuant Plate™.

The sequences used for the experiments were:

*#1 DNA hairpin:* t(Alexa555) gagcgGTATAGCCTAATTCGcgctct (BHQ-2) ctgtcactttctgag - 3'.

*#2 DNA input:* 5'-DNPtttttttttttCGAATTAGGCTATAC-3'

Strand #1 was terminally modified with Alexa555 and internally with BHQ-2 (black hole quencher 2).

*CYP2E1 assays.* CYP2E1 assay kit (Life Technologies) contained: Vivid CYP2E1 Substrate (EOMCC 7-(ethoxymethoxy)-2-oxo-2H-1-benzopyran-3-carbonitrile 0.1 mg), NADP<sup>+</sup> (10 mM in 100 mM potassium phosphate buffer pH 8.0), Vivid Regeneration System (333 mM glucose-6-phosphate and 30 U/mL glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate buffer pH 8.0) and microsomes from baculovirus-infected cells co-expressing CYP2E1, NADPH-cytochrome P450 reductase and cytochrome b5 (CYP2E1 Baculosomes 0.5 nmol in 100 mM potassium phosphate buffer pH 7.4).

*Inkjet printing fL-scale aqueous solutions on glass surfaces.* Droplets were dispensed on glass substrates by a Dimatix Materials Printer (DMP-2800, Fuji Film) at 40% Relative Humidity. This instrument was equipped with piezo-driven inkjet print cartridges, each with 16 nozzles 254  $\mu\text{m}$  spaced and 21.5  $\mu\text{m}$  (for 10 pL cartridge) or 10.5  $\mu\text{m}$  (for 1 pL cartridge) in diameter. Mineral oil droplets were dispensed by using conventional waveform at 5 kHz and 10 pL cartridges at temperatures of 33°C and at voltages of 28 - 30 V. An ensemble of 1000 oil droplets (droplet-to-droplet pitch of 5  $\mu\text{m}$ ) formed a single droplet of about 10 nL by coalescence. We fabricated mineral oil microarrays in a 4  $\times$  2 format of 10 nL drops with a spacing of 1 mm. About 5  $\cdot$  10<sup>3</sup> fL-scale aqueous droplets with a drop-to-drop pitch of 40  $\mu\text{m}$  were injected in the oil drops by using our designed waveform at 1.2 KHz and 1 pL cartridges at room temperature and at voltages of 12 V - 14 V. Water-in-oil droplet microarrays were fabricated on coverslip glasses (Corning 24  $\times$  50 mm, 0.13–0.16 thick), functionalized following reported protocols.<sup>[25]</sup>

*Confocal microscopy and FLIM Analysis.* Confocal images of fL-droplets were acquired in one channel with an Olympus FluoView1200 confocal laser scanning microscope (Olympus) using a 60x 1.35 NA objective. We used 515 nm laser to excite the Alexa 555 dye of DNA

hairpin. The bandwidth of the emission filter used for the red emission channel is 550-650 nm. We collected data in the photon-counting mode. The scan area was 512x512 pixels, the pixel size was set at 0.05  $\mu\text{m}$  and the scan speed was 4  $\mu\text{s}/\text{pixel}$ . Images were analysed by Image J software (1.46r version). For FLIM analysis,  $256 \times 256$  pixels image stacks were sequentially acquired using a Leica TCS SP5 confocal scanning microscope with a 63x 1.4 NA objective (Leica Microsystems, Germany), scan frequency was 400 kHz. For the CCVJ, 2-Photon excitation was set at 830 nm and the detection range at 450-650 nm, while for the CYP2E1 catalysed reaction the 2-Photon excitation was set at 810 nm, the detection range at 440-520 nm. Leica “white laser” was used for the excitation of FITC, Alexa 647, Atto655-BTN, DNA hairpin and was set at 488 nm, 647 nm, 655 nm, 515 nm, respectively. Fluorescence decay was initially evaluated with the SymPhotime Analysis software; FLIM images were analyzed using the SimFCS 3.0 program (Laboratory for Fluorescence Dynamics, University of California, Irvine, CA) to calculate the fluorescence lifetime with the phasor method.<sup>[31]</sup> Droplets confocal images for CYP2E1 assays were acquired with the Leica TCS SP5 confocal scanning microscope; fluorescence intensity values of bulk solutions and fL-droplets were obtained by Image J software (1.46r version). FLIM on annular regions of fL-droplets were carried out by replicating the analysis on 6 different maps (each containing typically 6 droplets) to obtain a signal/noise ratio comparable to the one of HBDs. Fluorescence intensity and lifetime values have been averaged by at least 10 different droplets.

### Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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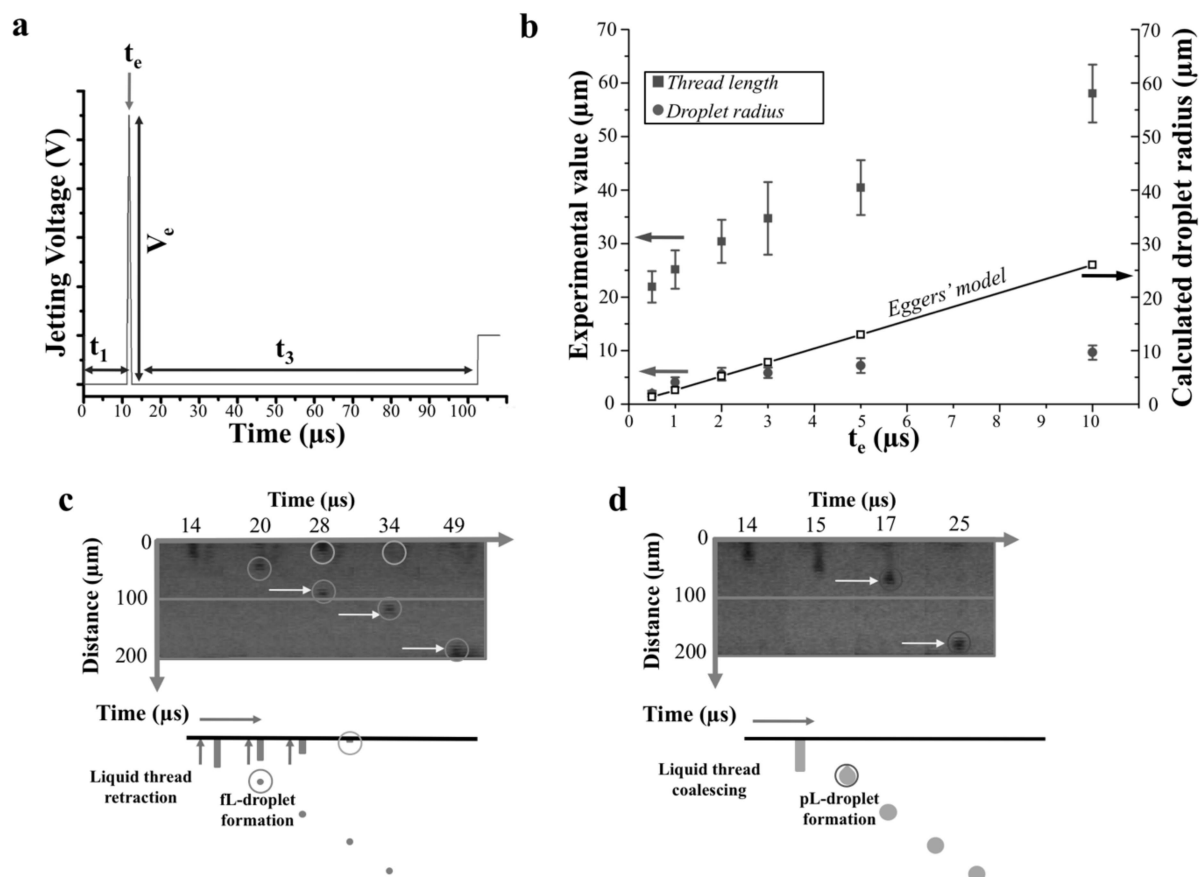
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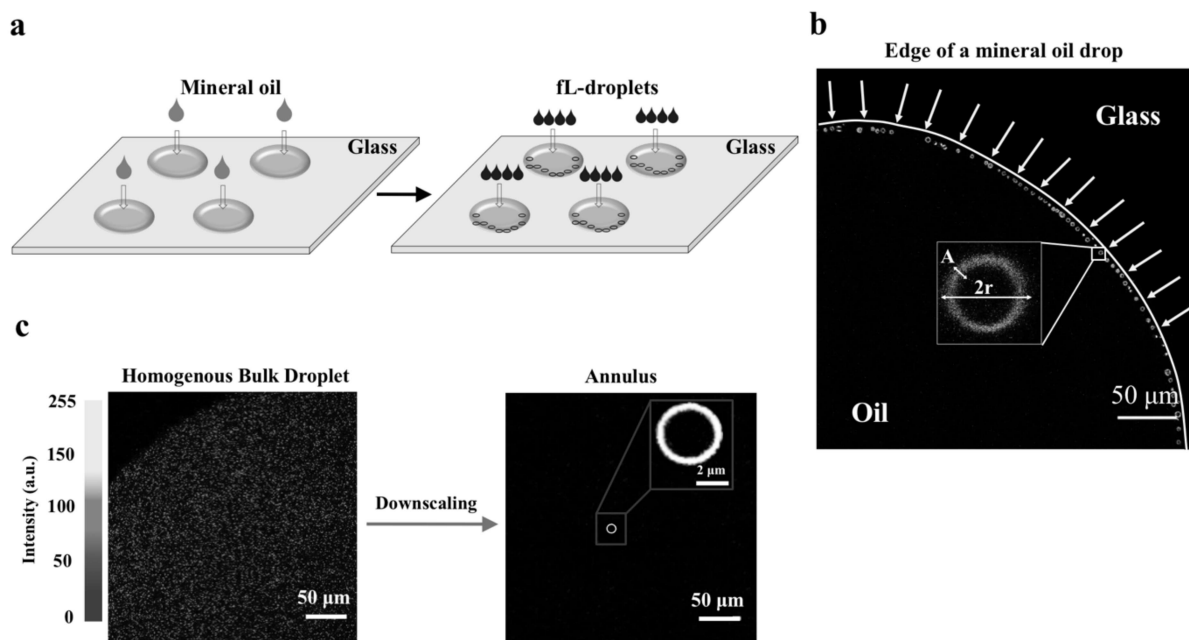
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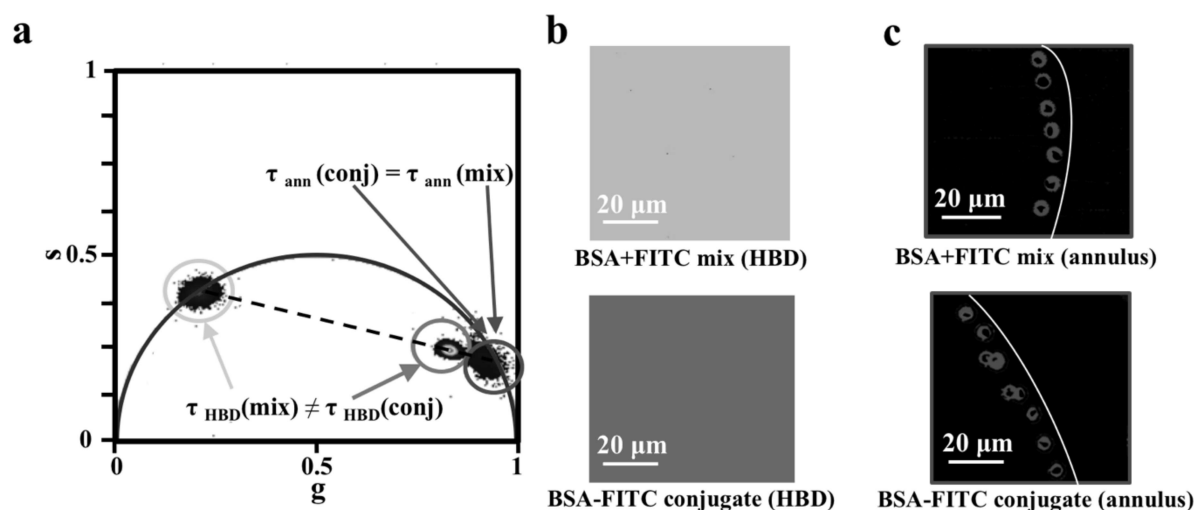
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**Figure 1. Aqueous femtoliter droplets by minimized inkjet printing ejection time. a)** Piezoelectric driving “spike” waveform for ejecting fL-droplets. The ejection phase is equal to  $0.5 \mu\text{s}$ . The pulling phase lasts  $90 \mu\text{s}$  ( $t_e$  the time of droplet ejection,  $t_1$  and  $t_2$  the time intervals for liquid pulling back into the nozzle,  $V_e$  the jetting potential at droplet ejection). The intervals  $t_1$  and  $t_3$  permit to pull back the liquid tongue in the nozzle orifice. **b)** Experimental values of the droplet radius at pinch-off (green circle) and thread length (red squares) along with the Egger’s model predicting the minimal droplet radius values (hollow squares) plotted as a function of  $t_e$ . Each experimental value is the average of five different droplets. **c-d)** Stroboscopic images of the droplet (water solution of  $4 \mu\text{M}$  Atto655 labelled Biotin,  $0.03\%$  F-108,  $0.05\%$  Tween20) pinching-off at the nozzles respectively for  $12 \leq V_e \leq 14$  and  $15 \leq V_e \leq 40$ . The schemes below show the droplet formation process at the fL-scale and at the conventional pL-scale, respectively. For the fL-scale, a pinch-off occurs after about  $20 \mu\text{s}$  (cyan circle), whereas the liquid tail remains attached at the nozzle and is pulled back after  $34 \mu\text{s}$  (orange circle), allowing reducing the droplet volume below the nozzle size. The pL-scale droplet formation results by the liquid thread coalescing into a single nozzle-sized droplet.

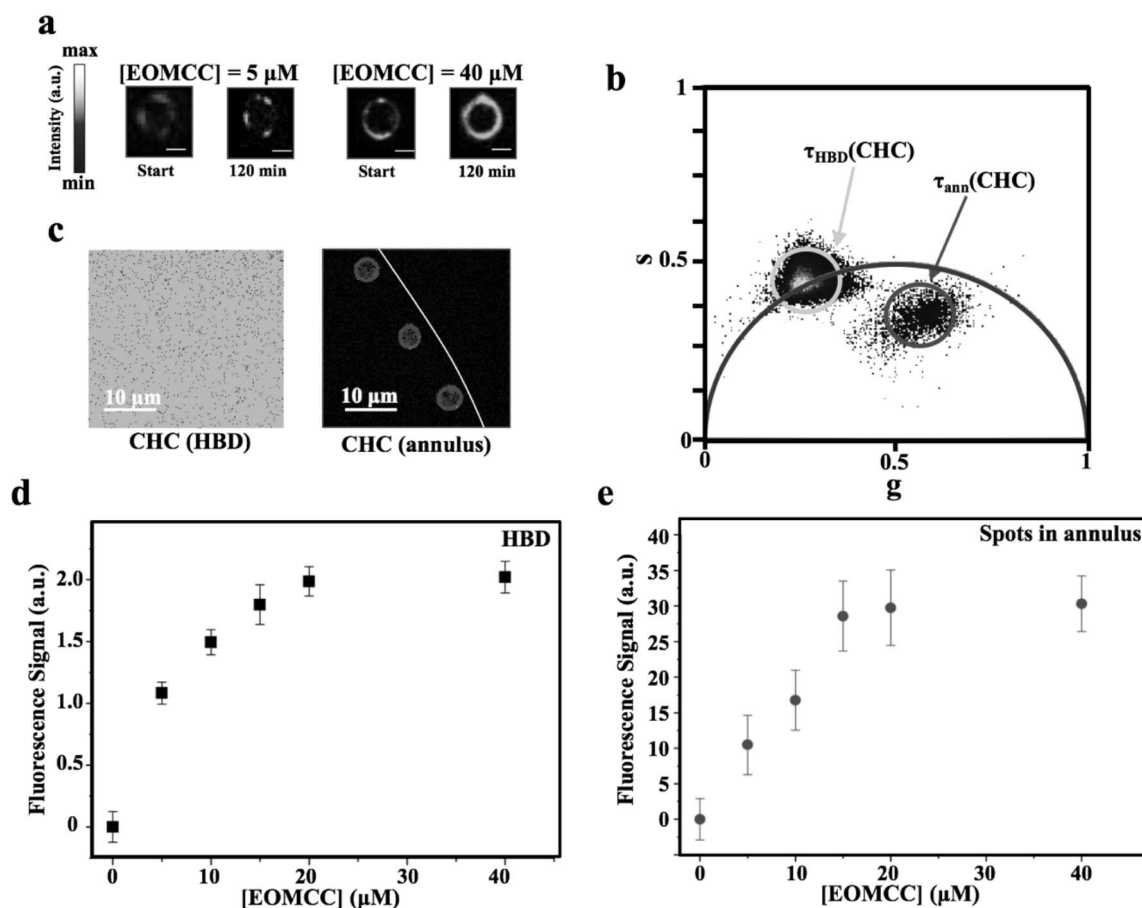


**Figure 2. Autonomous assembly of annular crowded confined structures in fL-droplets.** **a)** Injection of fL aqueous droplets (blue) produces micrometer droplets at the edge of the mineral oil drop arrayed on glass. **b)** Representative fluorescence images of a quasi-regular pattern of fL-droplets (containing 10 nM Alexa647) at the edge of a non-fluorescent mineral oil drop. Each optical section of the fL-droplets shows a fluorescent annulus at its perimeter. The inset shows a single fL-droplet along with its diameter ( $2r$ ) and the annulus thickness ( $A$ ). The white curve marks the oil drop edge. **c)** The formation of a fluorescent annulus (40 nM Atto655 labelled biotin) is observed by downscaling to fL-droplets and gives rise to an about 3.4-fold increase of molecular concentration with respect to HBDs.

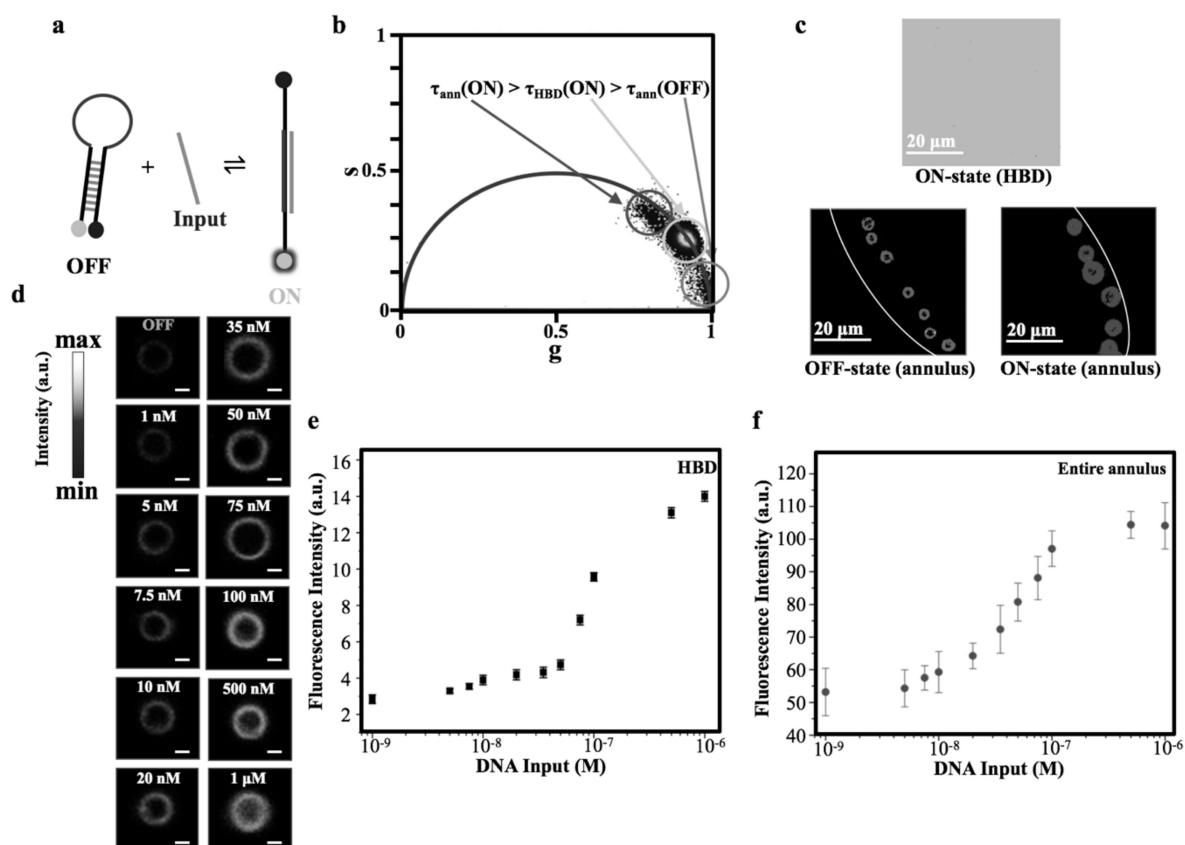


**Figure 3. FLIM analysis of BSA-FITC systems.** **a)** Phasor plot representing the fluorescence lifetime ( $\tau$ ) distributions in the annuli of fL-droplets for a BSA/FITC mixture (BSA 1.2  $\mu$ M, FITC 10  $\mu$ M; yellow cursor) and BSA-FITC conjugates (BSA-FITC 10  $\mu$ M; red cursor). **b)** Fluorescence lifetime selection maps for HBDs containing BSA/FITC mixture (orange) or BSA-FITC conjugates (magenta) with pixel signals showing lifetime distributions selected in the phasor plot by the circular cursors of the corresponding color. **c)** Fluorescence lifetime map in the annuli of fL-droplets coloured in red for both the BSA/FITC mixture or the BSA-FITC conjugate. The lifetimes are highlighted in the image by the same colour of the

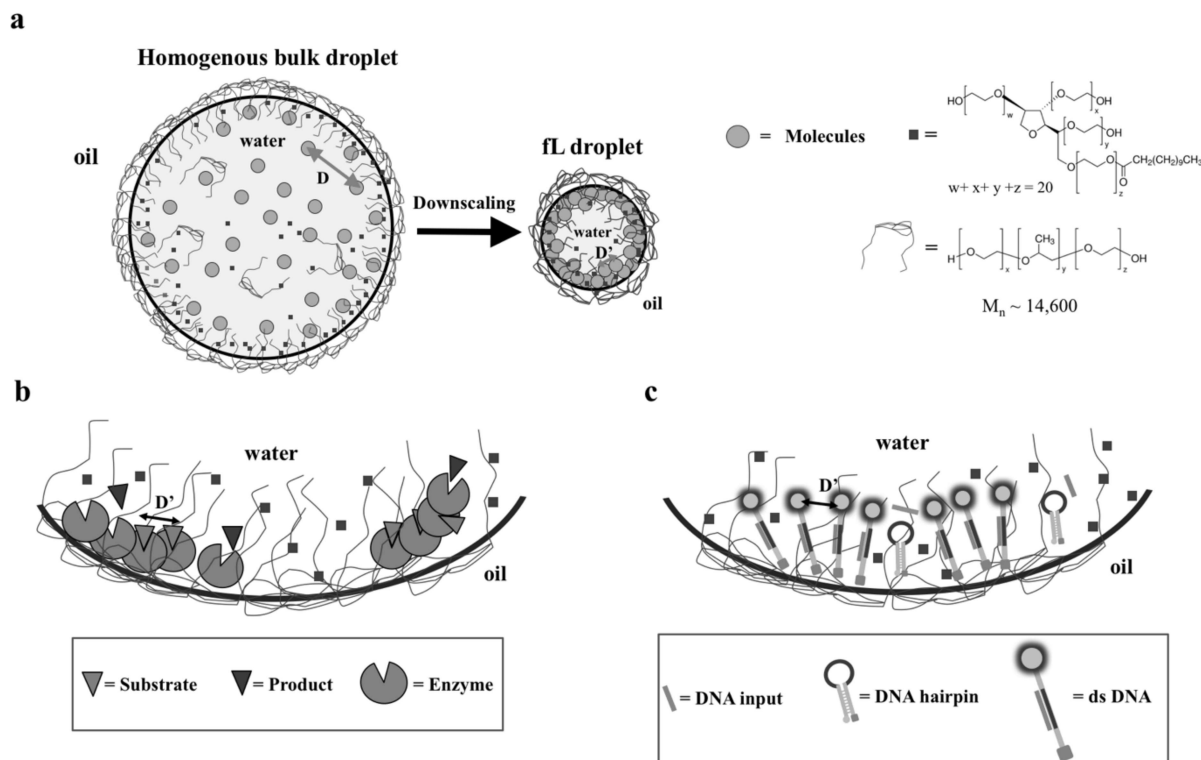
ursors in the phasor plot, in order to spatially separate pixels where different fluorescence lifetimes are measured. The white curve marks the oil drop edge.



**Figure 4. Model mitochondrial reaction in the annuli giving heterogeneous distribution of reaction products.** **a)** Heterogeneous distribution of CHC-rich spots at the annulus for two selected EOMCC concentrations (5  $\mu\text{M}$  and 40  $\mu\text{M}$ ) at the start of the reaction and after 120 minutes. Scale bar is equal to 2  $\mu\text{m}$ . **b)** The CYP2E1-catalysed reaction shows a single exponential fluorescence lifetime distribution in HBDs (orange cursor) and a more complex lifetime distribution within the annuli (red cursor) which can be ascribed to the presence of the two CHC species (undissociated and dissociated) as a result of water molecules depletion in the annulus. **c)** Corresponding fluorescence lifetime map for the samples in **b)** (orange colour for HBDs; red for annuli). The white curve marks the oil drop edge. CHC fluorescence in HBDs (black squares in panel **d**) and in the spots structures observed in the annuli (red circles in panel **e**) as a function of the EOMCC concentration. Note that in the case of annuli, the reported [EOMCC] values stand for the printed concentration of EOMCC in the fL droplet.



**Figure 5. DNA hairpin reactivity in confined annuli.** **a)** DNA hairpin switching from the OFF-state to the double helix ON-state by the action of a DNA target input. **b)**  $\tau$  distributions of DNA in ON-state in HBDs (orange cursor) and in the annuli of fL-droplets (red cursor). DNA in OFF-state shows a detectable  $\tau$  (magenta cursor) only when confined in the annuli. **c)** Fluorescence lifetime maps uniformly coloured in orange in the case of the DNA in ON-state in HBDs. All the fluorescence lifetime pixels in the annuli are coloured in red for DNA in ON-state and in magenta for DNA in OFF-state. The white curve marks the oil drop edge. **d)** Fluorescence images of annuli containing DNA hairpin at 10 nM in presence of increasing concentration of the DNA input. Scale bar is equal to 2  $\mu\text{m}$ . Fluorescence intensity in HBDs (black squares in panel **e**) and in the annuli (red circles in panel **f**) as a function of the number of DNA input concentration. Note that in the case of annuli, the concentration values stand for the printed concentration of DNA input in the fL droplet.



**Figure 6.** Schematic representations of molecular crowded confinement through amphiphilic layers with embedded molecular systems by downscaling the size of stabilized water droplets: **a)** the transition from HBDs to fL-droplets triggers interfacial molecular assemblies with closer intermolecular distances ( $D'$ ); **b-c)** restricted regions of fL-droplets surfaces showing discontinuous spots of enzymatic reaction products and DNA hairpins still responsive to molecular triggers under conformational changes, respectively. Note that, for sake of simplicity, the figures only show the adsorption of a single molecular layer at the water/oil interface, whereas in real systems it would consist of a  $\sim 500$  nm thick layer.

The table of contents entry should be 50–60 words long and should be written in the present tense and impersonal style (i.e., avoid we). The text should be different from the abstract text.

A mild inkjet-printing approach allows for aqueous droplets downscaling to the femtoliter scale, triggering the formation of self-selected submicrometer-thin molecular or biomolecular structures at the water/oil interface. Molecular probes jointly with fluorescence lifetime ( $\tau$ ) imaging allows to thoroughly characterize this artificial environment, revealing unique features (reduced water content, molecular crowding, molecular proximity, spatial localization) that closely mimic sub-cellular crowded compartments.

**Keyword: Sub-cellular scale compartments**

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**Printing life-inspired sub-cellular scale compartments with autonomous molecularly-crowded confinement**

ToC figure

