

## *In Situ* Lipid Dip-Pen Nanolithography Under Water

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**Summary:** Lipids form the structural and functional basis of biological membranes, and methods for studying their self-organization in well-defined nano- and micro-scale model systems can provide insights into biology. Using lipids as an ink for dip-pen nanolithography (lipid DPN) permits the rapid nanostructuring of multicomponent model lipid membrane systems, but this technique has so far been limited to air. Here we demonstrate that lipid DPN can be carried out under water with single tips or parallel arrays. Using the same tip for deposition and imaging in aqueous solution permits imaging of self-spreading lipid bilayer spots *in situ* and quantification of the nanoscale spreading kinetics in real time by means of lateral-force microscopy. Furthermore, using fluorophore-labeled phospholipids, we directly observed, by confocal laser scanning microscopy, a two-phase (oil in water) meniscus formed around the contact point between the DPN tip and surface, gaining insights into the mechanisms of the ink transport. The methods described here provide a new tool and environment for high-resolution studies of lipid nanodynamics and molecular printing processes in general. SCANNING 32: 15–23, 2010. © 2010 Wiley Periodicals, Inc.

**Key words:** dip-pen nanolithography, phospholipid, *in situ* imaging, atomic force microscopy, confocal optical microscopy

### Introduction

Scanning-probe technologies have unique potential for directly characterizing and functionalizing interfaces with biological materials at ultrahigh resolution because of their ability to function in humid air and aqueous environments (Alessandrini and Facci 2005; Loos 2005). For example, this ability permits direct observation in real time of dynamic processes in reconstituted biomolecular systems with molecular resolution *in situ*. Examples include the direct observation of DNA supercoiling (Lyubchenko and Shlyakhtenko 1997), DNA–protein interactions (Jiao *et al.* 2001; Neaves *et al.* 2009), and structure and dynamics of lipid membranes (Goksu *et al.* 2009; Iijima *et al.* 2009). Furthermore, the direct physical interactions between the atomic-force microscopy (AFM) tip and the sample allow local surface modification, allowing use of the AFM tip for scanning-probe lithography (Tseng *et al.* 2008). A variety of mechanisms can be used to structure the surfaces with an AFM tip, for example local application of mechanical (Hirtz *et al.* 2009), electrical (Maoz *et al.* 2000), or thermal energy (Cannara *et al.* 2008).

Dip-pen nanolithography (DPN) differs conceptually from other scanning-probe lithography in that, rather than delivering energy to the surface, DPN directly delivers materials to the surface from an ink-coated AFM tip in a molecular printing

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process (Braunschweig *et al.* 2009; Piner *et al.* 1999; Salaita *et al.* 2007). The constructive, bottom-up nature of DPN makes it scalable by means of parallel arrays and permits the integration of multiple materials on the same surface (Hong and Mirkin 2000; Lenhert *et al.* 2009; Salaita *et al.* 2006; Wang *et al.* 2008). This capability, combined with DPN's ability to function under hydrating (humid) conditions, suits it particularly well for the direct fabrication of multicomponent biomolecular nanoarrays, as has been demonstrated for DNA (Demers *et al.* 2002), protein (Lee *et al.* 2003), and phospholipids (Lenhert *et al.* 2007).

A convenient capability of DPN, shared by most scanning probe-based lithography processes (as well as electron beam lithography), is the possibility of simultaneous *in situ* imaging and writing with the same probe. For example, in an early DPN study, Piner *et al.* (1999) observed monolayer growth *in situ* by scanning the same area of a gold surface repeatedly with an alkanethiol-coated tip at high resolution to observe the self-assembled monolayer growth dynamics. When this method was applied to the deposition of poly-DL-lysine hydrobromide on mica surfaces, the nucleation and growth dynamics of polymer crystals could be both observed and controlled at a submicron scale inaccessible by other methods (Liu *et al.* 2005).

The mechanism of ink transport in DPN is based on the spontaneous formation of a micro/nano-scale liquid meniscus between the tip and surface. In ambient air, for example, such a meniscus naturally forms by capillary condensation (Xiao and Qian 2000), and ink molecules such as alkanethiols can move either through this meniscus (Piner *et al.* 1999) or on its surface (Nafday *et al.* 2006). The shape of such a microscopic liquid meniscus is determined by the interfacial energies involved, as well as the local geometry and kinetic factors (de Gennes 1985; Marmur 1993). Such a meniscus has been directly observed by environmental scanning electron microscopy (Weeks and DeYoreo, 2006; Weeks *et al.* 2005) and its size shown to be correlated with alkanethiol ink transport rates (Nafday and Weeks 2007). Evidence also shows that both the amount of alkanethiol ink on the tip and the surface roughness of the tip's coating affect the ink transport rate, an effect that has been explained in terms of the dissolution rate of the ink in the meniscus (Giam *et al.* 2009). In the case of mixed thiol inks, phase separation has been observed and discussed in terms of ink solubility in the meniscus and its surface (Salaita *et al.* 2005). Demonstration of the role of humidity in the partitioning of these mixed inks provides further evidence for the role of the meniscus in the transport of alkanethiols (Nafday and Weeks 2006). In cases where the ink itself is fluid

(Huang *et al.* 2009; Lenhert *et al.* 2007; Senesi *et al.* 2009), however, the role of the condensed water meniscus is less clear, as the ink itself is a fluid and could be expected to form a meniscus in the same way as on more traditional pens (Fan *et al.* 2000), pin spotters (Dufva 2005), or micropipettes without the need for capillary condensation.

Micropipettes are similar to DPN in capabilities, and although they do not offer the resolution or registration of DPN, they have the advantage that they function while immersed (Bruckbauer *et al.* 2002, 2003; Lewis *et al.* 1999; Suryavanshi and Yu 2007; Taha *et al.* 2003). Although nanofountain pens, which combine concepts of DPN with those of micropipettes (Moldovan *et al.* 2006), have recently been used in parallel and multiplexed to increase scale, and materials have even been delivered to living cells in air (Loh *et al.* 2009), carrying out such experiments under water remains a challenge, as the concepts of DPN are so far limited to air. Micro-contact printing of water-insoluble thiols has been carried out under water for some time now, for example, to minimize ink transfer from the stamp through the vapor phase by the use of reactive thiol spreading on a gold surface (Xia and Whitesides 1995). Furthermore, AFM tips coated with a bilayer in solution by means of a vesicle-fusion technique have been used to exchange lipids with a preformed supported lipid bilayer on the surface under water (Carlson *et al.* 2000).

Phospholipids are the main structural and functional component of biological membranes, and patterning them on subcellular scales is useful for *in vitro* biological investigations into the role of membrane patterning and dynamic adhesion processes in cell function (Chen *et al.* 1997; Irvine *et al.* 2007; Lee *et al.* 2002; Mossman *et al.* 2005; Sekula *et al.* 2008; Wu *et al.* 2004). Furthermore, surface-supported bilayers under water are useful as model membrane systems (Sackmann 1996), and phospholipid interactions with surfaces can provide insights into lipid nanodynamic processes (Nissen *et al.* 1999, 2001; Radler *et al.* 1995; Sanii and Parikh 2007). Fluid phospholipids such as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) are particularly well suited as inks for DPN in air because their viscosity and corresponding ink transport properties can be controlled by relative humidity (Lenhert *et al.* 2007). The use of humidity-controlled ink fluidity, as well as the possibility of dispersing functional lipids into the ink, permits multiplexed DPN with a variety of fluorescent and functional phospholipids simultaneously from different tips in parallel arrays (Sekula *et al.* 2008; Wang *et al.* 2008), but so far *in situ* AFM characterization has not been possible with lipid DPN because the capillary forces present during imaging in contact mode in air disrupt the lipid patterns. Furthermore, the relative roles of

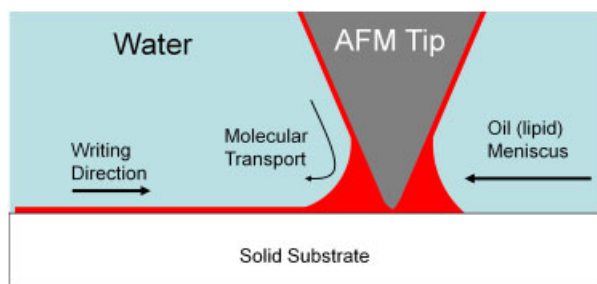


Fig 1. Schematic drawing of the concept of *in situ* lipid dip-pen nanolithography (DPN). An atomic-force microscopy (AFM) tip is coated with a water-insoluble (e.g. phospholipid) ink. Upon immersion in aqueous solution, and contact with a solid substrate, an oil meniscus forms in water, the size of which determines the transport rate of the ink between the tip and the surface.

capillary-induced meniscus condensation and humidity-induced lipid fluidity in lipid DPN remain unclear. Because biological molecules such as phospholipids carry out their biological function under water, the ability to pattern and image them simultaneously *in situ* under water by DPN would be particularly desirable.

Here we report a demonstration that lipid DPN can be carried out under water on clean surfaces with single tips or parallel arrays. Figure 1 shows the concept of *in situ* lipid DPN. An AFM tip is coated with a water-insoluble ink (in this case DOPC) and immersed in aqueous solution. Upon contact with the surface, an oil-in-water meniscus forms, allowing the lipid ink to be transported to the surface. Although the term meniscus is most commonly used to refer to a curved liquid/vapor interface in contact with a solid surface, the same interface-physics concepts apply when any two immiscible fluids are used (Brochard-Wyart 1995), for example oil and water (Danov *et al.* 2006). Using the same tip for deposition and imaging in solution (where capillary condensation no longer disrupts the pattern during imaging) permits quantification of the spreading kinetics of lipid bilayers. Furthermore, using fluorophore-labeled phospholipids, we directly observed the oil–water meniscus at the DPN tip by *in situ* confocal laser scanning microscopy (CLSM). This direct observation provides insights into the mechanisms of lipid ink transport and a novel way to observe and control nanoscale lipid dynamics in solution.

## Materials and Methods

### Materials

DOPC and fluorophore-labeled lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (18:1 lissamine rhodamine PE)

were purchased from Avanti Polar Lipids, Alabaster, AL. Chloroform solvent was HPLC grade (Sigma-Aldrich, St. Louis, MO). Nanopure water with a resistivity of 18.2 M $\Omega$  cm was used.

### DPN and *In Situ* AFM

DPN patterning and *in situ* AFM were carried out on a commercial instrument equipped with an environmental chamber (NScriptor, NanoInk, Chicago, IL) and a homemade fluid cell. One-dimensional tip arrays with 26 tips were used (Type F, side A26), as were single tips (Type A, both sides) and inkwells of type W4 and IWL-0021-01 (NanoInk). A crucial point to understand in the process of lipid-DPN in general is that the tips can be coated by phospholipids in a solvent-free manner, under conditions where the phospholipids themselves are fluid. The inkwells were filled with a chloroform solution of the phospholipid ink (1  $\mu$ l, 10 mM, doped with 1 mol% of the dye-labeled lipid in the case of doped inks). The solvent was allowed to evaporate completely (for at least 1 h under vacuum) before the tips were coated. Tips were inked by placement in contact with the inkwell and increasing the humidity to 70% (conditions under which the phospholipid becomes fluid) for at least 5 min. The microfluidic inkwells permitted selective delivery of inks to different tips in a parallel array. Tips were placed in contact with the surface before immersion in solution. *In situ* AFM imaging was carried out in contact mode with the same tip for writing and imaging and fast scan rates between 5 and 10 Hz. Glass coverslips (Thermo Scientific, Waltham, MA) were used as substrates and were treated with oxygen plasma (20 sccm, 100 mTorr, 30 s) before use. Transport rates were measured and recorded in real time with the InkCal function of the NanoInk software. A first-order exponential decay function was fit to the data with Origin 6.

### Optical Microscopy

Fluorescence microscopy was carried out on an inverted TE 2000 fluorescence microscope (Nikon, Tokyo, Japan) and Zeiss LSM510 (Zeiss, Oberkochen, Germany). Patterns on the surface were aligned for imaging by means of alignment marks scratched onto the glass surface. Water was added to the samples during imaging of the tips by pipetting into homemade wells cut from thin films of cured Poly-(dimethylsiloxane) (PDMS Sylgard 184, Dow Corning, Midland, MI) placed on the patterned glass coverslips. *In situ* confocal microscopy was carried

out with ink-coated tips (type F), and the cantilever chip was glued to a glass surface with superglue so that the tips were in contact with the surface. After the tips were immersed in solution, the glue bond to the glass was broken with tweezers so that the tips could be moved manually to a fresh location for observation of changes in the meniscus over time.

### Experiment 1

As a first test of the ability to write with lipids under water, every second tip in a parallel array of tips was coated with phospholipids; the uncoated tips were left as negative controls, as shown in Figure 2A and B. The freshly coated tips were placed in contact with the surface ( $O_2$  plasma oxidized glass) and immersed in pure water. The tips were then lifted from the surface and moved to fresh areas for patterning.

### Experiment 2

Once the appropriate conditions for reliably immersing the tips in solution had been established AFM could be carried out in liquid for *in situ* calibration and quality control (Haaheim *et al.* 2005), as shown in Figure 3. Lateral force microscopy (LFM) of areas in contact with coated tips for various lengths of time was carried out with that same tip. The tips were programmed to touch the surface for varying lengths of time, and the contact area was then scanned with a quick-scan rate of 5 Hz, which minimized continued writing during imaging.

## Results

In order to carry out lipid DPN reliably under water, one must first thoroughly coat the tips in air,

place them in contact with the surface, and then add the aqueous solution while the tips are still in contact with the surface. The lipid meniscus formed in air between the tip and surface is able to withstand immersion. Attempts to immerse the tips in solution before contact with the surface was established and were occasionally successful but not nearly as reproducible. The lipid ink was presumably washed away from the apex of the tip upon immersion, when the tip was not in contact with the surface.

### Experiment 1

Coated tips in contact with the surface for lengths of time ranging from 10 to 50 s left fluorescent spots with different radii in the patterning area, whereas no fluorescence was observed in the areas touched by the uncoated control tips (Fig. 2C). These results demonstrate that patterning only takes place from coated tips and that no cross-contamination occurs between neighboring tips, for example by transfer of the lipid ink through the surrounding solution rather than by direct contact. Furthermore, the use of fluorophore-labeled ink provides a rapid and high-throughput method of quality control and ink identification.

### Experiment 2

Figure 3A shows the LFM image resulting when the tips in Experiment 2 were programmed to contact the surface for 10, 20, 40, and 80 s. Fluorescence micrographs of the same areas are shown in Figure 3B. There, the higher-intensity spots can be seen, as well as a lower-intensity square, which corresponds to the scan area shown in Figure 3A. *In situ* LFM imaging permits direct measurement of the ink transport rate, as is commonly done for

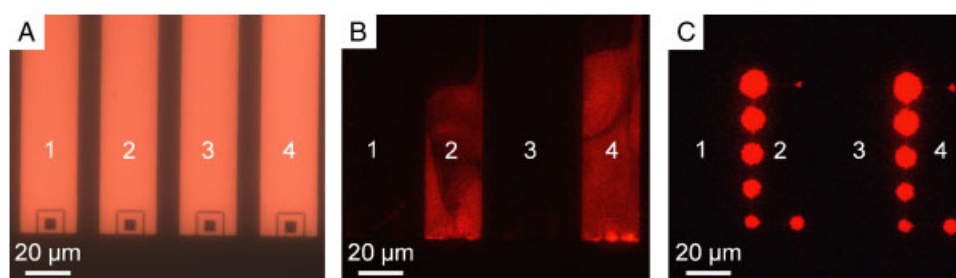


**Fig 2.** Parallel DPN carried out under water. Every second tip in a parallel array was coated with a phospholipid ink from inkwells, and the array was then used to produce patterns at different contact times. **(A)** Bright field image of the cantilevers. The pyramidal tips are visible as small dark squares at the ends of the cantilevers. **(B)** Fluorescence micrograph of the coated tips after writing (and removal from aqueous immersion for fluorescence imaging). Only every second tip was coated, and fluorescence is only observed from those tips, indicating that no cross contamination occurred during immersion. **(C)** Patterns drawn with the same cantilever array, still immersed. Tips were placed in contact with the surface for different amounts of time (ranging from 10 to 50 s), and the lipids spread only from the coated tips to form fluorescent circles.

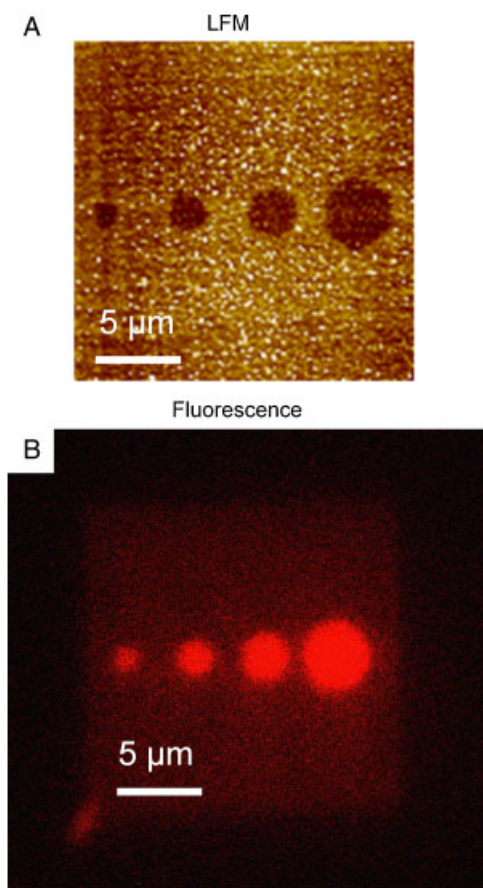


**Fig 3.** *In situ* AFM and *ex situ* fluorescence microscopy of lipid spots produced by DPN under water. **(A)** Lateral force micrograph (LFM) of lipid spots deposited at different tip contact times (10, 20, 40, and 80 s). The image was obtained with the same AFM tip used to generate the patterns. **(B)** Fluorescence micrograph confirming that the ink was deposited only where the tips came in contact with the surface.

alkanethiols in air (Jang *et al.* 2001). For this purpose, patterns like that in Figure 3A were used. The dot area was measured with commercially available software (Haaheim *et al.* 2005) and plotted against tip contact time to yield the transport rate as the slope of the line in units of  $\mu\text{m}^2/\text{s}$ , as shown in Figure 4A (which corresponds to the image in Fig. 3A). The transport rate was reproducibly observed to decrease over 3 h under water. Figure 4B shows a case in which the half-life of ink transport rate was 26 min. During the first 20 min after immersion, the transport rate was too high to measure *in situ*, as even a short contact time resulted in spreading beyond the scan range, and the high transport rate precluded imaging even at maximum scan rates of 10 Hz. The decrease in transport rate over time was fit to a first-order exponential decay function, as shown in Figure 4B. For investigation of the mechanisms of the ink transport under water, CLSM was used to examine the lipid-coated tip in contact with the surface before and after immersion in water, as shown in Figure 5. For this purpose, the chip was placed in contact with the glass surface and fixed in place with superglue. The fluorescent oil-in-water meniscus could clearly be seen in air, as shown in Figure 5A and B and took shape that would be expected for such a meniscus, for example from environmental scanning electron micrographs taken of a water meniscus forming at the contact point between an AFM tip and a surface (Weeks *et al.* 2005; Weeks and DeYoreo 2006). Upon initial immersion in aqueous solution, this meniscus was observed to expand quickly to form a large multi-lamellar vesicle (data not shown). When the tip was moved to a fresh area of the sample about 20 min

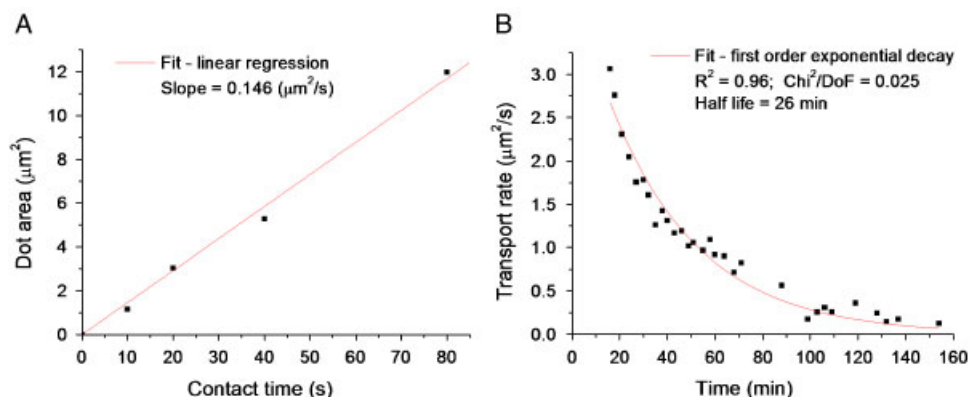


**Fig 4.** Decay of the ink transport rate over time. **(A)** Transport rates were determined from *in situ* LFM measurements and plotting the dot area against contact time. The data shown in (A) correspond to the LFM image in Figure 3A. The slope of the linear regression (fixed at the origin) defines the transport rate in units of  $\mu\text{m}^2/\text{s}$ . **(B)** A plot of transport rates measured *in situ* after immersion in aqueous solution. The time of immersion is defined here as time = 0. The transport rate under water reproducibly decreases over time scales on the order of hours, and a first-order exponential decay function is fit to the curve and reveals a half-life of 26 min.

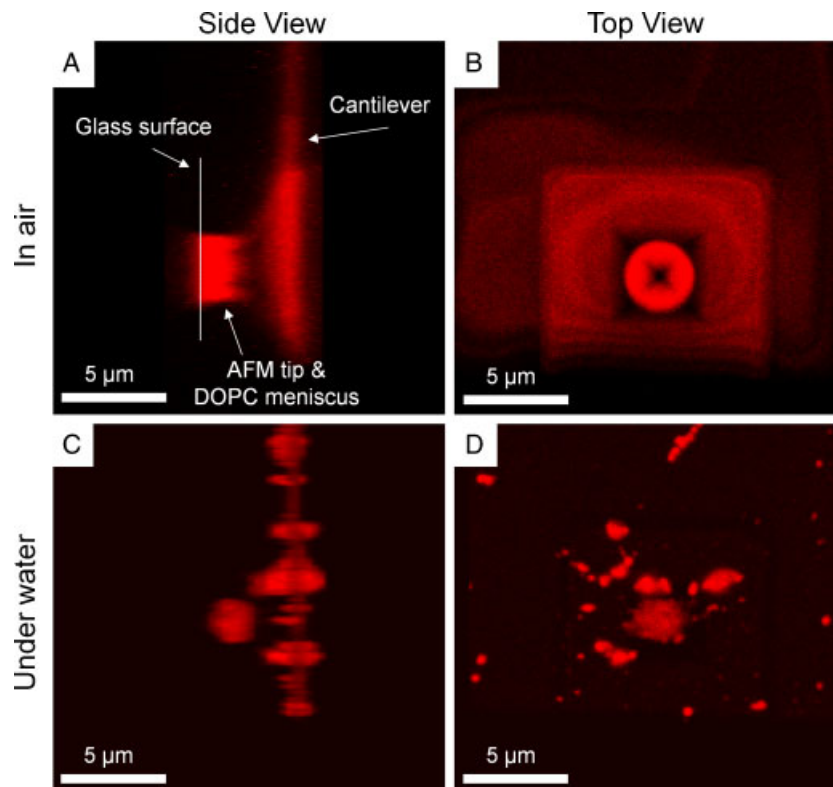


Fig 5. Projections through three-dimensional confocal image stacks of lipid-coated tips in contact with a glass surface. (A) Side view and (B) top view of the tip in contact with a glass surface. The fluorophore-labeled lipid meniscus can be clearly seen and has a contact diameter of about 3 μm. (C) Side view and (D) top view of the tip still in contact with the glass surface, under water after 20 min, with a contact diameter of about 2 μm.



Fig 6. Confocal section (red) overlaid with a bright-field image (gray scale) taken after ~1 h under water. The meniscus is still visible at the contact point of the tip on the left as a red spot, although, at a diameter of less than 1 μm, it is significantly smaller than it was initially.

after immersion, an oil–water meniscus like that shown in Figure 5C and D remained visible under water.

After longer immersion times, the size of the lipid meniscus was observed to decrease. For example, Figure 6 shows a significantly smaller meniscus after

about 1 h of immersion. Although the initial contact diameter of the meniscus shown in Figure 5 was 2–3 μm, after 20 min of immersion it had decreased to less than 1 μm. This observation suggests that ink depletion from the lipid meniscus is the mechanism behind the decay in transport rate shown in Figure 4.

## Discussion

A wealth of evidence indicates that the transport of alkanethiols in air is mediated by the presence of a water meniscus in air, but the role of the water meniscus in lipid DPN has been less clear. Although such a meniscus is known to be present in air, the fluidity of phospholipids also depends on the degree of hydration and therefore humidity, which empirically appears to influence the transport rate. Therefore, two mechanisms could explain the transport of phospholipid inks. First, lipids may be transported through a condensed water meniscus, and second, the fluid phospholipids themselves might form a meniscus at the surface contact point of the AFM tip. Because an air–water meniscus cannot exist under water, and we have directly observed the lipid meniscus both in air and under

water by CLSM, the data shown here provide evidence that the latter mechanism dominates ink transport in the case of phospholipids. That is, the ink does not necessarily move through a condensed aqueous meniscus if the ink itself is fluid. This observation may provide further insights into the mechanisms by which other DPN inks such as thiols dissolve (Giam *et al.* 2009) and phase separate (Nafday and Weeks 2006; Salaita *et al.* 2005) in the condensed water meniscus, as well as a perspective on transport of fluid inks (Huang *et al.* 2009; Lenhert *et al.* 2007; Senesi *et al.* 2009).

Although much work has addressed the qualitative explanation of the mechanisms of ink transport, obtaining quantitatively reproducible transport rates on different tips in parallel DPN remains a challenge. Evidence from the literature indicates that the amount of ink on the tip (Giam *et al.* 2009), the shape of the tip (John and Kulkarni 2007), the patterning time (Hampton *et al.* 2005), and the size of the condensed water meniscus (Nafday and Weeks 2007; Piner *et al.* 1999) all affect the transport rate. Our results further suggest that the geometry of the ink meniscus may be the factor that determines ink transport rates. Although the transport rates shown here for DPN under water are not stable over time, a rate of decay can be determined for the ink. Knowledge of this rate can extend experiment time to approximately 3 h, during which calibrations can be corrected accordingly, as compared with the several days during which lipid DPN in air can be carried out without re-dipping. Furthermore, it provides a method of systematically changing the transport rate from a single tip for experiments that may benefit from varying only this parameter. Because ink depletion appears to be the mechanism for the instability in the transport rates, the combination of principles from DPN and fountain pens or micropipettes for *in situ* lipid DPN could probably produce stable patterning and increase the reproducibility of transport rates.

Lipid spreading on solid surfaces has been well studied by time-lapse fluorescence microscopy (Nissen *et al.* 1999, 2001; Radler *et al.* 1995; Sanii and Parikh 2007). The spreading kinetics of a lipid bilayer on hydrophilic surfaces or of a monolayer on hydrophobic surfaces can be quantitatively described by means of adhesion theory as a balance between the spreading force and the resistive drag. *In situ* lipid DPN under water is a novel method of investigating nanoscopic structure and dynamics of these self-spreading molecularly thin films. Because phospholipids are fundamental components of cell membranes, *in situ* lipid DPN is especially promising for the investigation of dynamic model membrane-surface interactions. Finally, carrying out DPN under water provides a practical advantage in

DPN experiments for certain applications, because controlling the concentrations of materials in an aqueous solution or a lipid mixture is much easier than controlling atmospheric conditions. The under-water patterning methods and *in situ* CLSM as well as the insights into the mechanisms of ink transport in molecular printing should also be applicable to complementary methods such as micro-contact printing (Kumar and Whitesides 1993) and polymer pen lithography (Huo *et al.* 2008).

## Conclusions

We have developed a method for DPN under water based on using the water-insoluble phospholipid DOPC as an ink. The method can be reliably carried out provided that the tip and substrate are immersed, whereas the freshly coated AFM tip is in contact with the surface. This development makes possible *in situ* AFM imaging of the spread-supported lipid bilayer membranes as they form on the surface and quantification of the transport rates in real time. Investigation of the ink-coated tip in contact with the surface by CLSM reveals the formation of a lipid meniscus, and the size of this meniscus is correlated with the ink transport rate. In conclusion, *in situ* lipid DPN provides a new environment for studies of lipid nanodynamics.

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