

Multi-Ink Pattern Generation by Dip-Pen Nanolithography[®]

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Summary: Multiplexed patterns of hydrogels and phospholipids with fluorescent dyes are accomplished by dip-pen nanolithography[®] (DPN[®]). For example, four different dye-labeled hydrogel dot arrays are DPN[®] patterned within $50 \times 50 \mu\text{m}^2$ area, and two different dye-doped phospholipids dots and letters with less than $1 \mu\text{m}$ line-width are also DPN[®] patterned. We demonstrate that multi-ink patterns with precise alignment are able to be printed by DPN[®] within a micron-scale. Moreover, this multi-ink DPN[®] patterning methodology can be extended to delicate bio-materials printing in a subcellular scale with accurate positioning control. SCANNING 32: 24–29, 2010. © 2010 Wiley Periodicals, Inc.

Key words: dip pen nanolithography, multiplexing, poly(ethylene glycol), phospholipids, nanolithography platform (NLP)

Introduction

Dip-pen nanolithography[®] (DPN[®]) is one of the well-known nano-fabrication tools. It has been demonstrated to pattern soft and hard materials on various substrates. Examples include metals, semiconductors, and insulators (Agarwal *et al.* 2003; Basnar *et al.* 2006; Bruinink *et al.* 2005; Ginger *et al.* 2004; Hampton *et al.* 2005; Hyun *et al.* 2002; Jung *et al.* 2003; Li *et al.* 2001; McKendry *et al.* 2002; Mirkin 2007; Nelson *et al.* 2006; Noy *et al.* 2002; Nyamjav and Ivanisevic 2003; Pena *et al.* 2003; Salaita *et al.* 2007; Sheehan and Whitman 2002). In a typical DPN[®] process an AFM tip is used as a

pen to transport ink materials onto a substrate surface, which makes it possible to fabricate small features from sub 100 nm to over a micrometer in size. AFM tip-based small feature fabrication is the strength of the DPN[®] technique in comparison with other fabrication tools, such as micro array, micro stamping, and photolithography.

Recently, mass production of nano-features using parallel DPN[®] tip-arrays has been developed (Salaita *et al.* 2005, 2006). For a more elaborate application of DPN[®], one or more materials can be used as an ink library and can be DPN[®] patterned within a micron-scale region with precise alignment. Multi-ink DPN[®] has been used in the past to have a better understanding of a patterning mechanism and to improve resolution of multi-ink pattern fabrication (Hampton *et al.* 2006; Nafday and Weeks 2006; Nafday *et al.* 2009). An attempt of combining parallel tip-arrays DPN[®] with multi-ink DPN[®] has been reported (Sekula *et al.* 2008) for possible mass production of sub-micron-scale features that can be used large-scale-integrated protein chips. In order to detect more than one target proteins by an integrated protein chip, a multiplexed bio-material printing within the working area of the chip is highly desirable. Selectivity and sensitivity of the detection can be controlled by the number of materials and size of the features. This multiplexed protein pattern can also be used in cell binding or cell differentiating (in the case of stem cell) studies. For cell-binding studies, sub-micron features of protein pattern should be generated within the area of a single cell. In addition, sub-micron-scale patterning in large area will definitely improve sensitivity and selectivity of a protein chip, because an individual sub-micron feature has a small amount of proteins and a large number of features will have more chance to react with target proteins. For the aforementioned reasons, multiplexed bio-material patterning in sub-micron-scale has been studied (Arnold *et al.* 2004; Doh and Irvine 2006; Groves 2006; Mossman *et al.* 2005).

Herein, we demonstrate multiplexed hydrogel and phospholipid micron-structure patterning with precise registration. Hydrogels and phospholipids have been used as carriers for bio-materials DPN[®]

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patterning because of their easy DPN[®] capability and compatibility with bio-materials. In this study, various dye-labeled hydrogel and phospholipid inks were DPN[®] printed simultaneously. The whole process of multiplexed patterning was systemically carried out. (1) Multiple inks were added in a micron-channelled device that isolates individual inks with micron-scale separation. (2) The multiple inks were inked onto individual tips in a one-dimensional (1-D) tip-array. (3) Integrated printing of the multiple inks was carried out on a substrate with an accurate alignment and positioning using a CAD programmed nanolithography instruments. In the result, well-aligned and uniform multi-inked hydrogel and phospholipid dot-array patterns were obtained by printing and positioning of the table-top Nano Lithography Platform (NLP). Moreover, four different dyes-labeled 2×2 dot arrays were DPN[®] patterned within $50 \times 50 \mu\text{m}^2$ area for proof-of-concept of a multiplexed bio-material template in a subcellular scale.

Materials and Methods

For multiplexed ink printing, we used a micro-well to ink on a parallel tip-array with fluorescence-dye-labeled carrier materials (Fig. 1). Hydrogels and phospholipids have been reported as the excellent

bio-materials carriers (Huang *et al.* 2009; Lenhert *et al.* 2007; Sekula *et al.* 2008). Poly(ethylene glycol) dimethacrylate (PEG-DMA) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) materials were chosen as hydrogel and phospholipid materials for the multiplexed ink printing, respectively (Fig. 1(a)). For the hydrogel multiplexed colored ink, fluorescent dyes dissolved in ethanol were mixed with ethanolic PEG-DMA solution. We balanced the volume mixture ratio between dyes and PEG-DMA solutions to exhibit similar fluorescence intensity at each dye-labeled ink. For the phospholipid inks, fluorescent dye functionalized DOPC, which was purchased from Avanti polar lipids, Inc. (Alabaster, AL), was diluted with pure DOPC to a concentration of 1% (Lenhert *et al.* 2007).

An example of a multiplexed inking procedure is shown in Figure 1(b) and (c). Three different fluorescence-dye-labeled hydrogel inks were filled in the micron-sized channels (Fig. 1(b)). Rhodamine and fluorescein isothiocyanate (FITC) were used as red and green-colored dyes, respectively. For an orange-colored dye, rhodamine and FITC dyes were mixed in 50:50 volume ratio. Red, green, and orange-colored hydrogel inks were placed twice into every third micro-channel from right to left. A 1-D tip-array (M-type, NanoInk, Inc., Skokie, IL) was dipped into the various dye-labeled ink filled in the micro-inkwells for inking on the tip-array (Fig. 1(c)).

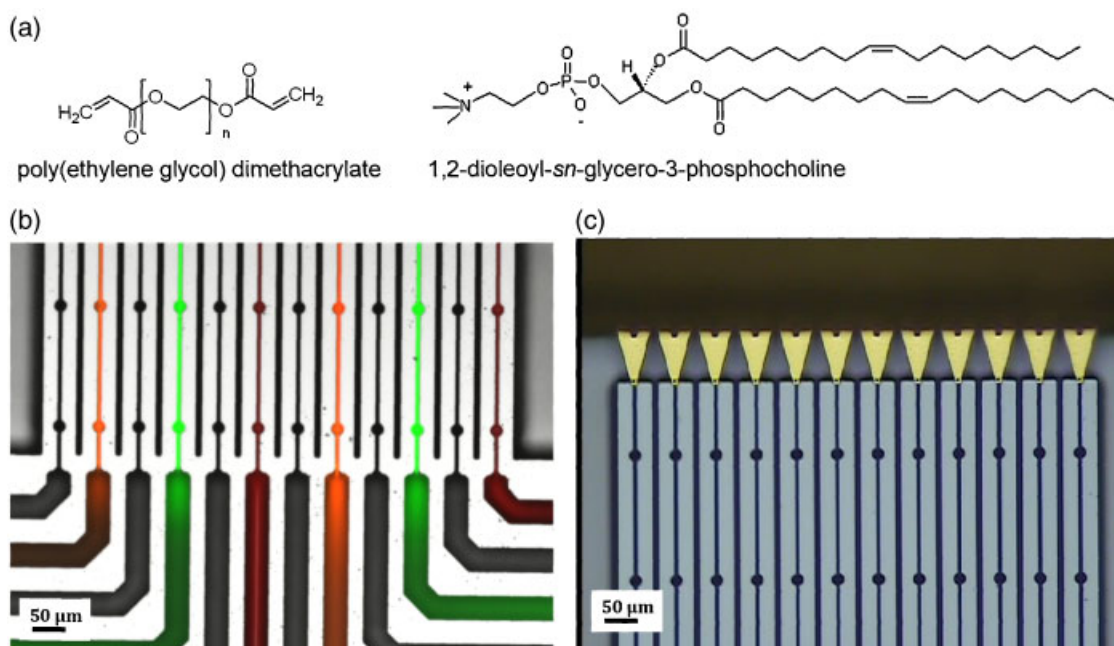


Fig 1. (a) Structure of carrier ink materials: hydrogel PEG-DMA and phospholipid DOPC. (b) Optical microscope images of a 12 channel micro-well with three different fluorescence colored inks in 6 channels. Red ink is rhodamine mixed PEG-DMA. Green ink is FITC mixed PEG-DMA. Orange ink is the mixture of red and green inks. Bright field and fluorescence images are merged together to display that inks are loaded in the micro-well without cross-contamination. (c) A captured optical microscope image when a tip-array is inking the inks in the micro-well. PEG-DMA, poly(ethylene glycol) dimethacrylate; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine. [Color figures can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DPN[®] printing of hydrogel ink was carried out on hexamethyldisilazane (HMDS) spin-coated on silicon substrates. A hydrophobic surface prevents spreading of PEG-DMA ink. Hydrogels spread out on hydrophilic surfaces because hydrogels are hydrophilic material. For phospholipid printing, DOPC ink was printed on an oxygen plasma-treated glass slide. Phospholipids are amphiphilic material, and can be DPN[®] printed on both hydrophobic and hydrophilic surfaces. The NLP2000[™] (NanoInk, Inc., Skokie, IL) was used for the multi-ink dot-array patterning because of its broad working stage area ($40 \times 40 \text{ mm}^2$). The spacing between individual tips in the tip-array is $66 \mu\text{m}$ (M-type, NanoInk, Inc., Skokie, IL). In order to closely print three-ink multiplexed patterns, the stage has to be allowed to move at least three times greater than the spacing between individually inked tips ($3 \times 66 \mu\text{m}$) in the X and Y directions. The broad working area in the NLP2000[™] is capable of closely printing more than three inks. The DPN5000[™] (NanoInk, Inc., Skokie, IL) system was used to print "CUBS" logo with two different dye-labeled phospholipid inks. Bitmap picture can be transformed into a dot-arrays map by the NanoWord[™] function in DPN5000[™] for printing the "CUBS" logo pattern.

Fluorescence optical microscopy images were measured by a ZEISS microscope (Axio Imager.Z1m, Thornwood, NY) with optical filters: cyanine 3 (Cy3), FITC, cyanine 3.5 (Cy3.5), and 4',6-diamidino-2-phenylindole (DAPI). Fluorescence optical microscope images were obtained with individual optical filters corresponding to the emission and excitation properties of the fluorescence dyes in the ink. Then fluorescence microscope images from the individual filtering were merged together in order to display the fluorescence images of multiplexed dye-labeled patterns in one image at the same time. Exposure time in seconds to the excitation light for individual fluorescence optical microscope imaging was individually adjusted in order to balance the intensity of the printed patterns.

Results and Discussion

Figure 2 shows hydrogel multi-ink DPN[®] patterns. Rhodamine- and FITC-labeled PEG-DMA 4×4 dot arrays were DPN[®] printed with a 1-D tip-array on HMDS coated silicon substrates (Fig. 2(a) and 2(b)). Figure 2(a) shows the scheme of the multi-ink printing. A 1-D tip-array (12 pens, M-type, NanoInk, Inc., Skokie, IL) was alternatively inked by rhodamine (red)- and FITC (yellow)-labeled PEG-DMA inks. The rhodamine-dye-labeled hydrogel was inked on every odd-numbered tip, and the FITC-labeled hydrogel was inked on every even-

numbered tip. A 4×4 dot array of rhodamine-labeled PEG-DMA (red) was printed by the first tip cantilever of the 1-D tip-array, and then another 4×4 dot array of FITC-labeled PEG-DMA (yellow) was patterned in this area by the second tip cantilever with precise positioning (Fig. 2(a)). Figure 2(b) shows red- and yellow-colored dot-array patterns with alternating order. The red, yellow, and red-dyes-labeled three 4×4 dot arrays with $5 \mu\text{m}$ of dot spacing (the left dot array in a red-yellow or yellow-red-colored unit-cell, the dot arrays are horizontally separated with $66 \mu\text{m}$ owing to the spacing of the tip-array) were printed three times from top row to bottom row with $15 \mu\text{m}$ spacing to the vertical direction, and then the yellow, red, and yellow-dyes-labeled 4×4 dot arrays (the right dot array in the unit-cell) were printed at the region with $20 \mu\text{m}$ offset to the right from the first array pattern area in a similar manner as the first array printing. The tip-array had traveled $46 \mu\text{m}$ ($66 - 20 \mu\text{m}$) to the left between the first and the second patterns. In the end, three rows of the well-aligned rhodamine- and FITC-labeled PEG-DMA multiplexed 4×4 dot arrays were obtained (Fig. 2(b)). The zoom-in image in Figure 2(b) shows the precise alignment between the first and second pattern: the distance between red- and yellow-dot arrays is $5 \mu\text{m}$. Diameter of red and yellow dots is ~ 0.9 and $\sim 1.2 \mu\text{m}$, respectively.

A more advanced multi-ink pattern of hydrogel was achieved and is shown in Figure 2(c). Four different dye-labeled hydrogel inks (PEG-DMA) are printed within a small area ($50 \times 50 \mu\text{m}^2$) (Fig. 2(c)). Rhodamine, FITC, Alexa347, and a mixture of rhodamine and FITC were used as red, green, blue, and orange dyes, respectively. Four arrays of 2×2 dots were DPN[®] patterned within $50 \times 50 \mu\text{m}^2$ area with $6 \mu\text{m}$ of dot spacing and $15 \mu\text{m}$ of array spacing using a 1-D tip-array (M-type, NanoInk, Inc., Skokie, IL). Four different dye-labeled PEG-DMA inks are inked on a 12-pen tip-array. The red, orange, blue, and green-dye-labeled PEG-DMA are inked on every first, second, third, and fourth tip cantilevers, respectively. In order to print one unit-cell of four dye-labeled hydrogel patterns (inset in Fig. 2(c)), a 2×2 dots array was four times DPN[®] patterned in the order of right-top, left-top, right-bottom, and left-bottom with a tip cantilever shifting to the printing area at left ($-x$ direction). In the inset in Figure 2, the 2×2 red-colored dots array is patterned at the right-top by the first tip cantilever, and the second tip is shifted to left-top corner with offset ($-87 \mu\text{m}$, $0 \mu\text{m}$) and prints the orange-colored dots array, and the third tip is shifted to right-bottom corner with offset ($-45 \mu\text{m}$, $-21 \mu\text{m}$) and prints the blue-colored dots array, and the fourth tip is shifted to left-bottom corner with offset ($-87 \mu\text{m}$, $0 \mu\text{m}$) and prints green-colored dots array, in rota-

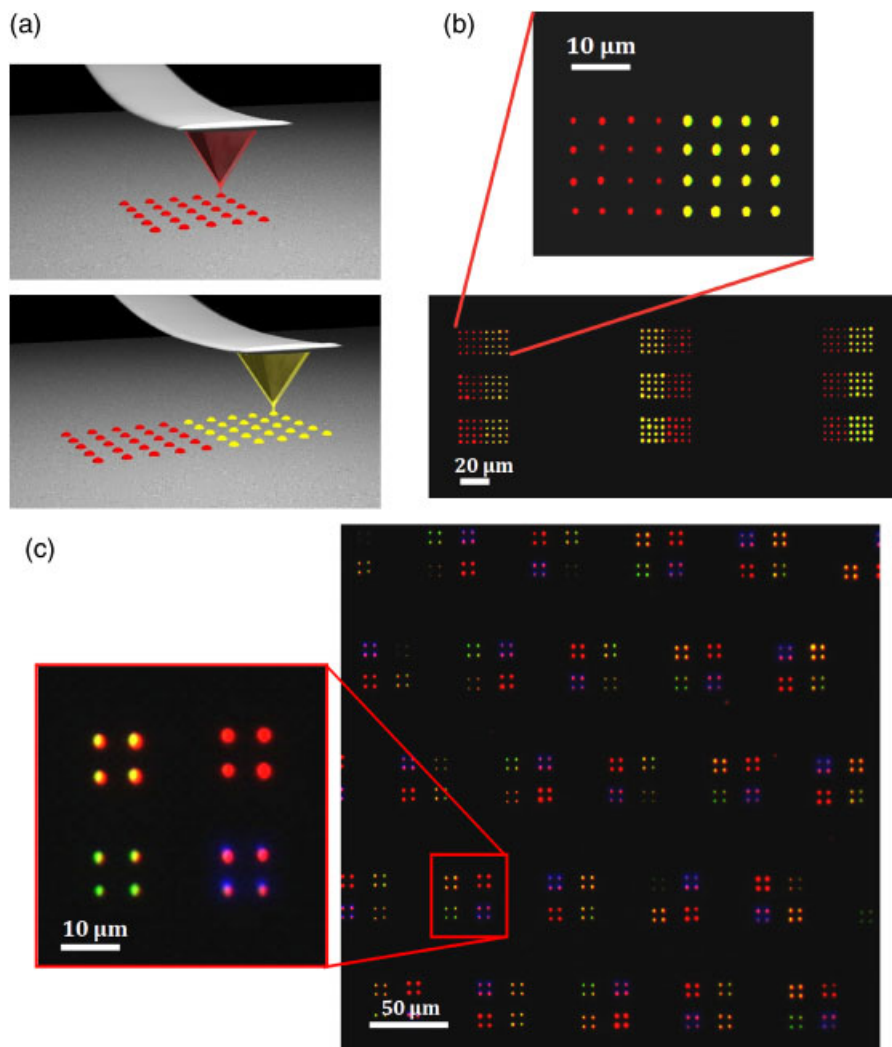


Fig 2. PEG-DMA hydrogel DPN[®] pattern. (a) Scheme of dot-array pattern with two-ink. (b) Fluorescence image of two-colored pattern using fluorescence-dye-labeled PEG hydrogel. Red color is from rhodamine, and yellow color is FITC with Cy3.5 filter. (c) A fluorescence microscope image of highly integrated PEG-DMA hydrogel multi-ink pattern. Four different dye-labeled PEG-DMA hydrogel patterns are printed within $50 \times 50 \mu\text{m}^2$ area. Inset shows mixture of rhodamine and FITC (orange), rhodamine (red), Alexa347 (blue), and, FITC (green)-labeled patterns in a clockwise direction from the left-top array. PEG-DMA, poly(ethylene glycol) dimethacrylate; DPN[®], dip-pen nanolithography[®]. [Color figures can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tion. Dot diameter of the arrays is 1–2 μm. In Figure 2(c) one row of 4 ink dot-array patterns was obtained by this one time of unit-cell patterning. Different color registries at every unit-cell of the pattern, which are shown in Figure 2(c), result from this combinatorial DPN[®] printing process. The colors of 2×2 dots arrays at the same position in a unit-cell are repeated in the order of red, orange, blue, and green (when one looks at a unit-cell from left to right in the same row), owing to the order of the dye-color in the tip cantilever. In addition, a unit-cell with the same color-registry displays a four-fold repeat in a row in Figure 2(c). After the fourth DPN[®] printing of a 2×2 dot array of one unit-cell, the inked cantilever is shifted to the vertical direction with a 50 μm offset and repeats a

printing of a unit-cell, routinely. Figure 2(c) shows the pattern repeated five times (five rows) of the unit-cell of the 2×2 dots array. The printing of an entire dot array was accomplished in 2 min. This result proves that highly integrated hydrogel nanostructures can be patterned by DPN[®].

Multi-ink patterns of phospholipids are shown in Figure 3. Figure 3(a) shows concentric square dot arrays of rhodamine (red)- and FITC (green)-labeled DOPC patterns. The green- and red-dye-labeled DOPC were inked on 1-D array pen (18 pens, 70 μm of tip spacing, E-type, NanoInk, Inc., Skokie, IL) in an alternating order. In this pattern, the inked tip cantilever was placed on the substrate aligned to the horizontal direction (the tip cantilever was parallel to the x -axis). Initially, the interior

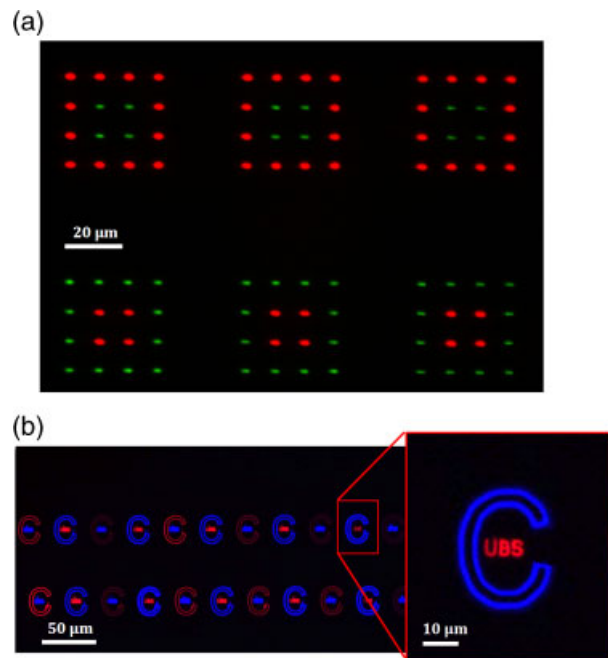


Fig 3. A fluorescence microscope image of DOPC phospholipid ink patterns. (a) Concentric square dot-array patterns with green (FITC)- and red (rhodamine)-dye-labeled phospholipid inks. (b) “CUBS” logo pattern with ~red (rhodamine)- and blue (DAPI)-dye-labeled phospholipid inks. [Color figures can be viewed in the online issue, which is available at www.interscience.wiley.com.]

2×2 dot array was printed three times with a $60 \mu\text{m}$ offset to the horizontal direction. This is followed by printing the outside concentric square dot array by a different colored dye-labeled tip after a shift ($10 \mu\text{m}$ to the left and $80 \mu\text{m}$ down). This pattern was also repeated three times with a $60 \mu\text{m}$ offset. Figure 3(a) shows the 4×4 dots array patterns of red- and green-dye-labeled DOPC with a highly accurate alignment. Dot spacing in the 4×4 dots array is $10 \mu\text{m}$ and both colored dot arrays are concentrically patterned without deviation. A tip dwell time of 0.01 s was used for printing each individual dot.

More advanced multi-ink patterns of phospholipids, patterned letters, were also carried out. The red- and blue-colored “CUBS” logo patterns are shown in Figure 3(b). Rhodamine (red)- and DAPI (blue)-labeled DOPC were used as the ink materials. The red- and blue-dye-labeled DOPC were alternately inked on a 1-D array pen (26 pens, $35 \mu\text{m}$ of tip spacing, F-type, NanoInk, Inc., Skokie, IL) in an alternating order. The DAPI-labeled DOPC was inked on every odd-numbered tip, and the rhodamine-labeled DOPC was inked on every even-numbered tip. The letter “C” was printed first, and then the letters “UBS” was printed next with the other dye-labeled tip cantilever. Between both printings, the tip cantilever was shifted to the horizontal direction ($50 \mu\text{m}$ offset to the left) and slightly to the

vertical direction ($3.5 \mu\text{m}$ offset to down) in order to print “CUBS” logo with two-color inks. Another row of “CUBS” logo patterns was printed to the bottom with $70 \mu\text{m}$ offset after printing the first row of “CUBS” logo patterns (Fig. 3(b)). Inset in Figure 3(b) shows the size of letter “C” and “UBS” as ~ 25 and $\sim 4 \mu\text{m}$, respectively. A dye-labeled DOPC pattern with less than $1 \mu\text{m}$ resolution was achieved: the pattern of “UBS” letters is clearly patterned with less than $1 \mu\text{m}$ line-width without any duplication (inset in Fig. 3(b)).

This multi-ink patterning methodology can be extended to other applications by broadening the ink library. Hydrogels and phospholipids are useful carriers because of their easy modification with other functional materials. For example, PEG can be DPN[®] patterned with nanoparticles, antibodies, or small molecules in the ink (Huang *et al.* 2009). DOPC has been used as a carrier for a protein DPN[®] patterning (Lenhert *et al.* 2007; Sekula *et al.* 2008). Moreover, the number of inks can be increased by developing the tip-array and the micro-inkwells. At this moment, 12 inks can be patterned with our methodology by the 12-pen array and 12-channeled micro-well (DNA micro-well, NanoInk, Inc., Skokie, IL). The writing time of multiplexed patterning is quite comparable to other printing methods. The entire pattern in Figure 2(c) was printed within 2 min. DPN[®] is capable of printing multiplexed hydrogel and phospholipid inks and can be extended to multiplexed protein printing with relatively fast writing times and precise alignment control.

Conclusion

In conclusion, we have demonstrated the multiplexed ink patterning in small area (subcellular size) with precise alignment and positioning. Hydrogel and phospholipid patterns are able to be multiplexed by up to four different inks at present, but it can be expanded more according to micro-channel and tip cantilever development. This multi-ink DPN[®] methodology can be used in highly integrated protein patterning, and can provide the template for the protein sensing and cell-binding studies.

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