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Application note on Miniaturized Assays – printing microarrays of phospholipids

Miniaturized assays - microarrays of phospholipids

Application note

Introduction

Development of miniaturized assays that allow for large test areas to be created and tested but require only small volumes of test analytes to be used, as often only small test sample sizes are available, are important steps towards personalized medicine. Developing such miniaturized biochips containing arrays of test molecules requires a methodology that allows the user to deposit molecules at high resolution and speed while maintaining functionality. Lipid dip-pen nanolithography (L-DPN) is an ideal technique to create such biologically active surfaces, and it has been successfully applied in the direct deposition of functional proteins as well as in the fabrication of biochemical templates for selective adsorption.

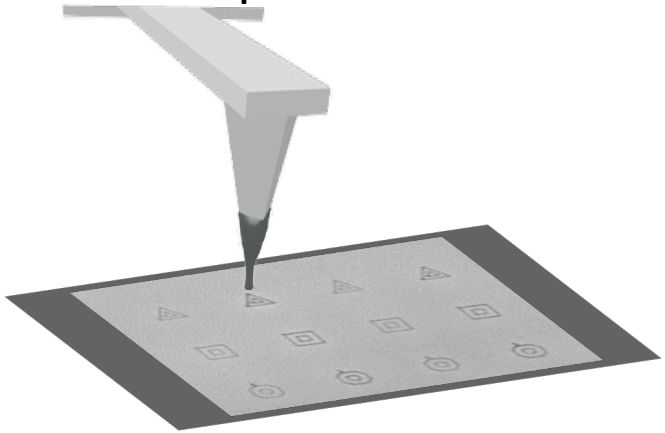
In this Application Note we describe the utilisation of L-DPN in the generation of arrays of the lipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) onto glass surfaces as a model system. L-DPN, using phospholipids as ink biomolecules, enables the decoration of solid supports with tailored patches of lipid membrane that can be used as *in vitro* membrane models. In addition, such arrays, when prepared with lipids that have reactive headgroups, can be applied for various applications ranging from site-specific protein binding and detection of antibodies to cell based assays.

Workflow

Application note

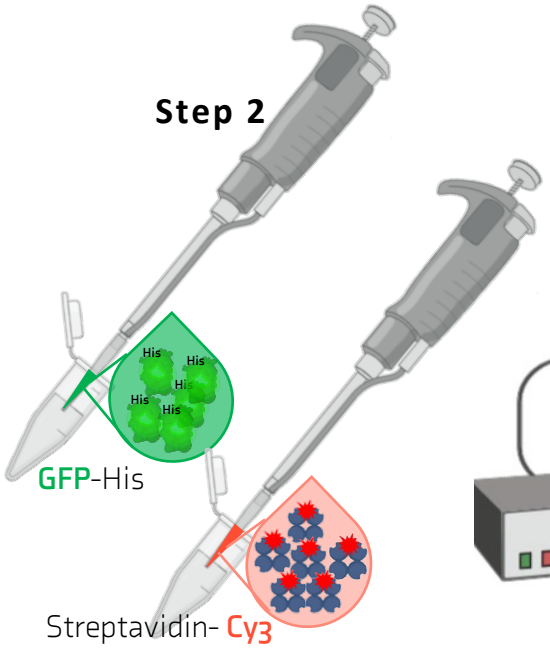
The workflow for preparing the graphene sensor is shown below and can be divided into three distinct steps:

Step 1



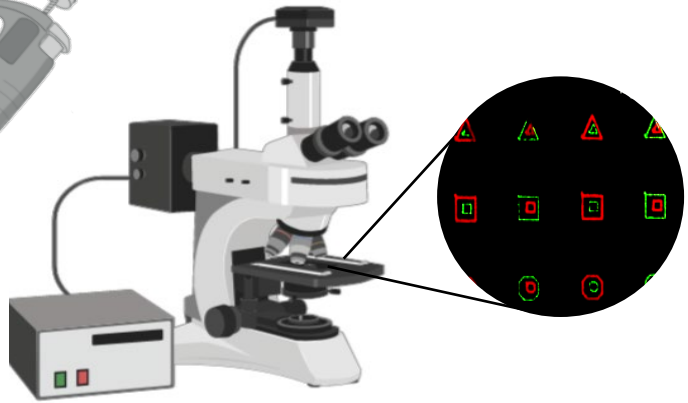
Printing lipids

Step 2



Analyte bonding/
Reaction with printed ink
(e.g. Proteins)

Step 3

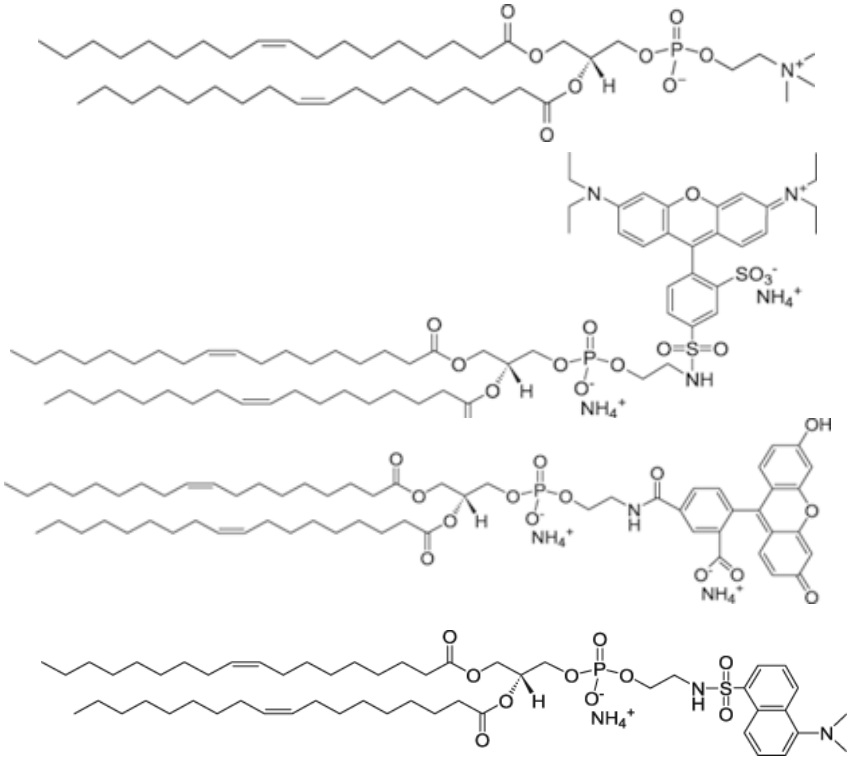


Detection of bound target molecules
(e.g. Fluorescence microscope)

Step 1 - Printing lipids

Application note

To prepare the inks for printing fluorescent lipid microarrays the following lipids were used:



18:1 (Δ9-Cis) PC (DOPC)
1,2-dioleoyl-sn-glycero-3-phosphocholine

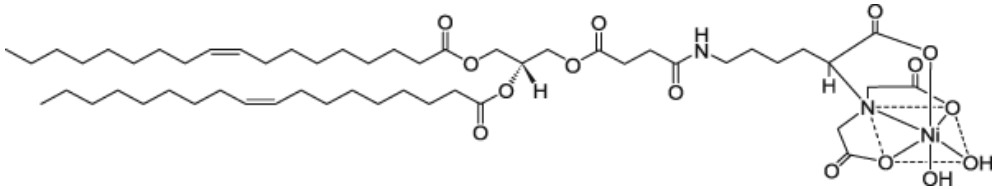
18:1 Liss Rhod PE
1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)

18:1 PE CF
1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein) (ammonium salt)

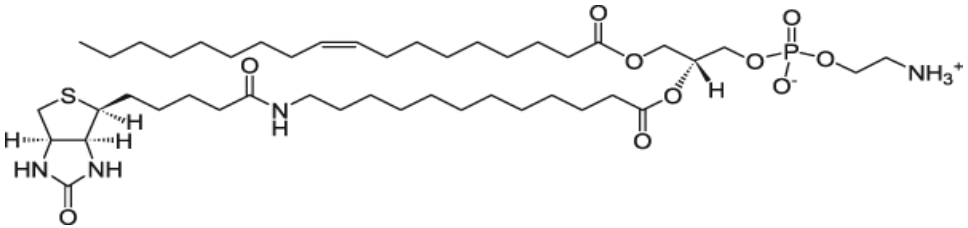
18:1 Dansyl PE
1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl) (ammonium salt)

Step 1 - Printing lipids

To prepare the inks for printing protein binding microarrays the following lipid with functional headgroups were used:

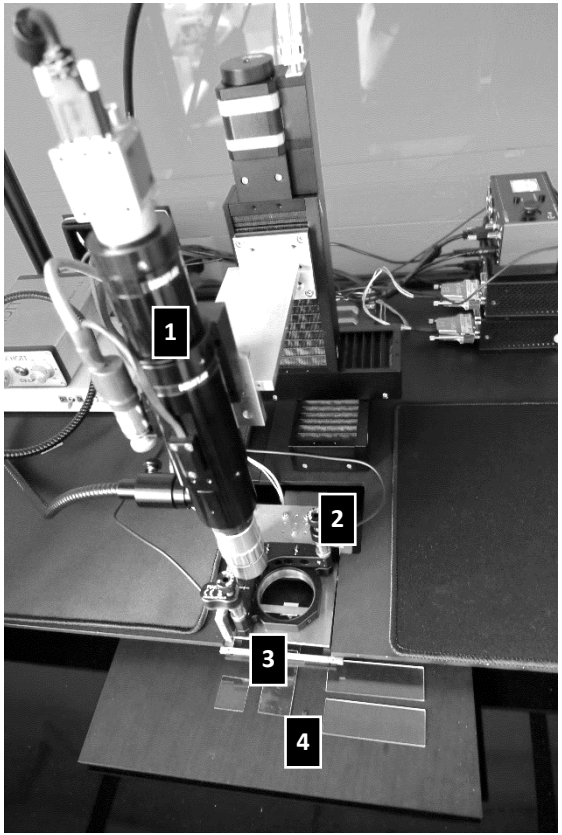


18:1-12:0 Biotin PE
1-oleoyl-2-(12-biotinyl(aminododecanoyl))-sn-glycero-3-phosphoethanolamine



18:1 DGS-NTA(Ni)
1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt)

Step 1 - Printing lipids



1 Microscope module for *in situ* process control (image and video aquisition)

2 High precision module for high resolution control over the printing/spotting process

3 Probe holder for mounting probes

4 Sample table for loading the substrates

All phospholipids used in the experiments described were supplied by Avanti Polar Lipids US, as a solution in chloroform.
The DOPC carrier solution (20 mg/ml) was admixed with appropriate amounts of fluorescently labelled lipids: Rhodamine, Dansyl or Carboxyfluorescein, to obtain a final 1 mol % concentration in DOPC. For printing functional arrays, NTA-Ni and Biotin lipids were admixed to DOPC at 10 mol% and 4 mol% respectively.

Lithographic set-up – Molecular Printer

All lipid writing processes were performed with M-type one-dimensional cantilever arrays (acs-t, USA). Appropriate sample inkwells (acs-t, USA), matching the M-type cantilevers, were loaded with 1.5 µl of the different phospholipid mixtures and then dried in a vacuum dessicator for 15 min. to remove the solvent (chloroform). The M-type cantilever array was inked in the inkwells for 10 min at a humidity of 70 % R.H. After inking, excess ink was removed by writing on a 'sacrificial' area on the sample substrate prior to printing onto the target area. Lithography on the glass target area was typically performed at humidity of 30% R.H, with a dwell time of 200 ms and a writing speed of 5 - 10 µm/s for lines.

Step 2 - Analyte binding

After the lipid writing process, the samples were prepared for binding the target molecules by:

- blocking with BSA (10 % in PBS, Sigma Aldrich) for 20 minutes,
- washing with PBS 3 x,
- incubating with target molecules (at 5 µg/ml final concentration):
  - Fluorescently labelled streptavidin in PBS (Streptavidin- Cy3, Sigma Aldrich) for 15 minutes,
  - Fluorescent GFP-His6x (protein kindly provided by Prof. Dr. Matthias Franzreb, KIT) for 30 minutes,
- washing again in PBS 3 x and in water 1 x.

The area functionalized with the biotin- or NTA-Ni- lipids are clearly observed by fluorescence after Streptavidin –Cy3 or GFP-His binding, whereas just the DOPC lipid patterns remain dark and serve as a negative control (data not shown).

Step 3 – Detection: Fluorescence microscopy

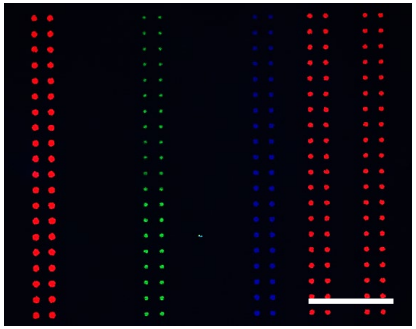
Fluorescence microscopy images were taken with a Nikon upright fluorescence microscope (Eclipse 80i) or Nikon inverted microscope TE2000, both equipped with a sensitive camera CoolSNAP HQ2 (Photometrics). The broadband excitation light source (Intensilight illumination) was combined with sets of filters (Texas Red, FITC, DAPI) to separate excitation and emission spectra, depending on the dye molecule used.

Step 3 – Detection:
Fluorescence microscopy of fluorescently labelled lipid arrays

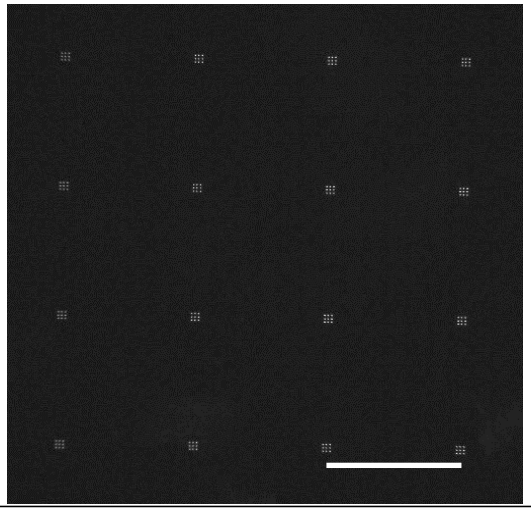
Application note

In L-DPN, an atomic force microscopy (AFM) tip coated with the desired lipid mixture is brought into contact with the target area on the substrate and directed over the surface in the desired pattern. The lipids transfer to the substrate surface is facilitated by a water meniscus that is formed under the controlled environmental humidity of the system. Lipids then self-assemble into membranes. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was used as the main carrier for the different phospholipid inks as it enables the writing of the lipids onto the substrate. To this carrier **1** mol% of Rhodamine, Dansyl or Carboxyfluorescein were added. The lipid mixtures are fluorescent and therefore are easily detectable for optical control of the writing outcome.

This approach can be utilized to fabricate test phospholipid arrays for optimizing the writing process or for testing the fluorescence of the admixed dyes in air or in various solutions (PBS, or low / high pH solutions). In the examples below fluorescence microscopy of lipid arrays was performed in air.



Lipid array composed of dots spaced with 20 µm and printed with DOPC admixed with **1** mol% of of Rhodaimne. Printing parameters: dwell time **200** ms, **30**% R.H. Scale bar equals **100** µm.

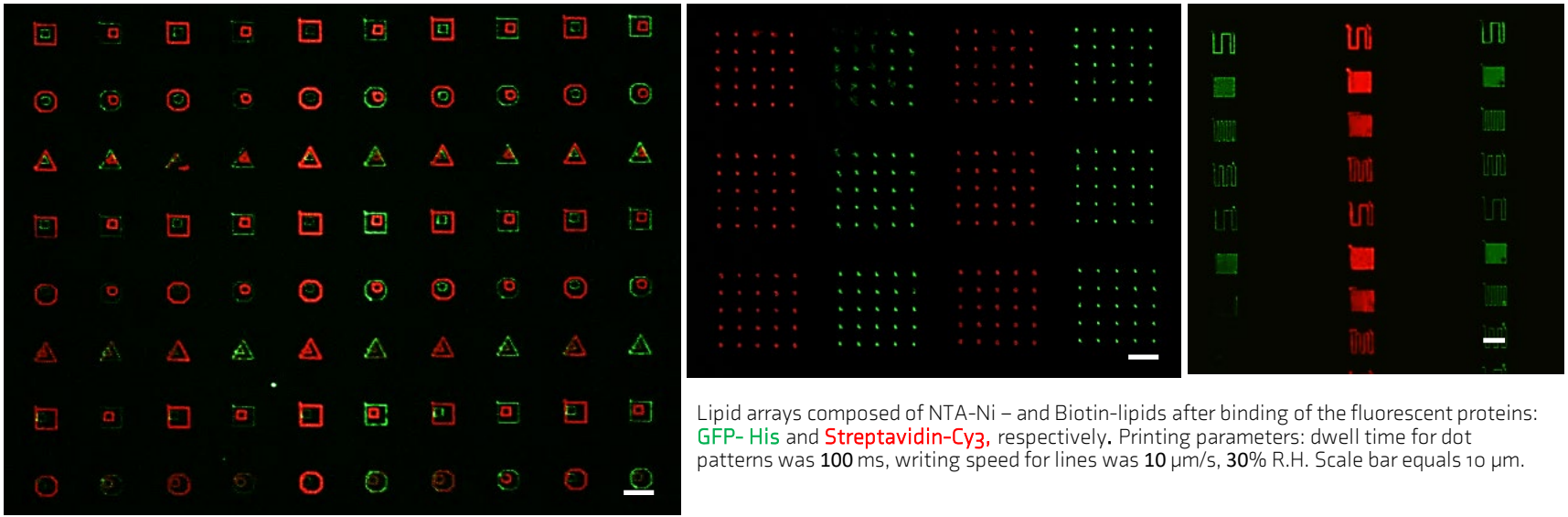


Lipid array printed with DOPC admixed with **1** mol% of of Rhodamine (red), Dansyl (blue) and Carboxyfluorescein (green). Printing parameters: writing speed **10**µm/s, **30**% R.H. Scale bar equals **100** µm.

Step 3 – Detection:
Fluorescence microscopy of functinal lipid arrays

Application note

In case of functional arrays,**10** mol% of NTA-Ni or **4** mol % of Biotin lipid were admixed to DOPC. After the writing process was completed samples were used directly for binding the target molecules (His-GFP and Streptavidin-Cy3, respectively). Fluorescence microscopy was performed in water. Detection of fluorescence in GFP and TexasRed channel confirmed successful binding of target proteins to the functional lipid patterns. This approach can be utilized to fabricate arbitrary functional protein (or antibody) arrays that can be used in next steps for cell culture experiments. Selected examples of functional phospholipid arrays present a range of patterns that can be created during the writing process. Such arrays can prove useful in addressing various questions on protein-lipid, protein-protein or protein-cell studies.



Lipid arrays composed of NTA-Ni – and Biotin-lipids after binding of the fluorescent proteins: GFP- His and Streptavidin-Cy3, respectively. Printing parameters: dwell time for dot patterns was **100** ms, writing speed for lines was **10** µm/s, **30**% R.H. Scale bar equals 10 µm.

Conclusions

Molecular patterning processes taking place in biological systems are challenging to study *in vivo* because of their dynamic behaviour, subcellular size, and high degree of complexity. Surface immobilization of bioactive molecules has drawn much attention in recent years, as it is a fundamental technique for the preparation of bioactive surfaces. *In vitro* patterning of biomolecules using lithography allows for simplification of the molecular patterning processes and detailed study of the dynamic interactions between lipids, proteins and cells. Parallel lipid dip-pen nanolithography (L-DPN) is uniquely capable of integrating functional biomolecules on subcellular length scales due to its constructive nature, high resolution, and high throughput. Phospholipids are particularly well suitable as inks for DPN since a variety of different functional lipids can be readily patterned in parallel. In the examples presented here DPN was used to spatially pattern multicomponent lipid arrays that are fluid and contain various amounts of fluorescent or functional (biotin or NTA-Ni) headgroups. Selective adsorption of functionalized or recombinant proteins based on Streptavidin or Histidine-tag coupling enables the semisynthetic fabrication of model peripheral membrane bound protein –phospholipid arrays. The biomimetic membrane patterns formed in this way can then be easily incorporated into various microfluidic systems and applied in miniaturized assays.

Selected literature:

Application note

- Sekula et al., Multiplexed lipid dip-pen nanolithography on subcellular scales for the templating of functional proteins and cell culture. Small, 2008, 4, 1785-1793
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- Mitsakakis et al., Convergence of dip-pen nanolithography and acoustic biosensors towards a rapid-analysis multi-sample microsystem. Analyst 2012, 137, 3076–3082
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- Bog at al., On-chip microlasers for biomolecular detection via highly localized deposition of a multifunctional phospholipid ink. Lab Chip, 2013, 13, 2701-2707
- Oppong et al., Localization and Dynamics of Glucocorticoid Receptor at the Plasma Membrane of Activated Mast Cells. Small 2014, 10, 1991-1998
- Liu et al., Development of Dip-Pen Nanolithography (DPN) and Its Derivatives. Small, 2019, 15, 1900564

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