Abschlussbericht

Kurzbezeichnung des Vorhabens

ChemoChips

Förderkennzeichen

16IN0219

Zuwendungsempfänger

TU-Ilmenau

Projektleiter

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Berichtszeitraum

2003-2006

Laufzeit des Vorhabens

01.09.2003 - 31.07.2006

Publications with relevance to the reported Project

1. A. Thete, G. A. Groß, J. M. Köhler, „Investigation of diffusion behavior of indicator dyes in hydro gel films for chemochip application using a microfluidic arrangement”,
   VDE Micro System Technique Congress Proceedings, 2005, M22

2. A.R. Thete, G.A. Gross, J.M. Koehler, “Microfluidic arrangement with an integrated micro-spot array for the characterization of pH and solvent polarity” Chemical Engineering Journal 135S (2008) pages S327-S332

3. A.R. Thete, T. Henkel, R. Göckeritz, M. Endlich, G.A. Gross and J.M. Koehler “A hydrogel based fluorescent micro spot array for the characterization of liquid analytes”
   Analytica Chimica Acta, Volume 633, Issue 1, 2 February 2009, Pages 81-89

Brief Project Summary

The “ChemoChip” project part of the TU-Ilmenau was defined in the project plan to following issues:

- Development of an opto-fluidic measurement setup
- Development of a appropriate measurement cuvette (in cooperation with IPHT-Jena)
- Characterization of the setup
- Investigation of sensoric thin films
- Exemplary analytic measurements

All scheduled project tasks were fulfilled in cooperation with the other Project partners. The overall concept of “ChemoChips” was proved for aqueous and non aqueous liquid analyte samples. Individual spot intensities as well as patterns of spot array were applied for analytic readout purposes. Following the received results are presented.

1. Instrumentation and experimental methods

The experimental work has been divided in to the three main aspects:

I) Working and adopting of commercial as well as specially developed instruments

II) Preparation and standardization of micro spot arrays

III) Experimental methods using optical and microfluidic techniques

The instrumentation plays role in the preparation, characterization, and readout of the chemochips. Following section covers the introduction to all instrumentation used in different departments of the work. The chip preparation materials, methods and microfluidic experimental details are covered from next section onwards.

1.1. Instrumentation

The instrumentation used can be categorized into commercial instruments and specially developed research instruments. This is summarized in the following table.

Table 1: Instrumentation summary applied for ChemoChip characterization

		Instrument	Manufacturer	Application purpose
Commercial instruments	1	Nano-Plotter	GeSim mbH (Dresden/ D)	array preparation, micro spotting of dyes or polymers as well as modifiers
	2	Atomic force microscope	Vecco Instruments (USA)	surface characterization short range polymer spot
	3	Fluorescence microscope Axioplan 2 imaging	Zeiss GmbH (Jena/D)	optical investigation of double layer polymer spots
	4	Reflection profilometer	UBM Inc. (USA)	double layer polymer spot characterization

Specially developed research instruments	5	Sensovation optical setup adapted to the microfluidic needs	Sensovation AG (Stockach/D)	Opto-fluidic System for fluorimetric imaging

1.2. Standard instruments

1.2.1. Nano-Plotter

The Nano-Plotter™ instrument is ideal to generate high-quality spots by non-contact microdispensing of sub-nanoliter volumes. The versatile "drop on demand" piezo technology allows the placement of very small drops onto various surfaces. The instrument was used to plot various types of pure as well as mixed dye solutions on polymer and glass surfaces. The high resolution robotic movements of the dispensing tip allows the compact, high density spot arrays using about 100 pL of sample volume. The dispensing is presented schematically in the figure 1. The tip use piezo actuation for the creation of droplet. This gives the possibility to create a very small volume droplet with high reproducibility and speed. The droplet of demand can be achieved by altering the number of droplet, piezo voltage, frequency and pulse width. The projection of actual droplet can be monitored using the high speed stroboscopic camera integrated in the spotting device itself (Fig. 1). In chemochips preparation a batch of 30 - 40 chips was spotted at a time to have identical spotting properties for all the chips. This helps to achieve better spot reproducibility. A few million spots can be generated once the pipette is filled.

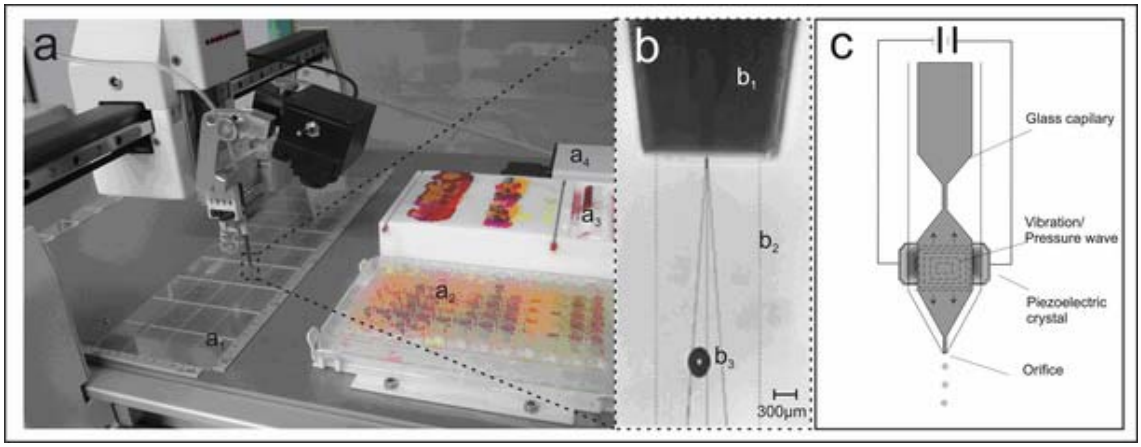


Figure 1: (a) Nano-Plotter used for spotting dye solution as a chemochip preparation step. a_1: chemochips for spotting a_2: MTP filled with dye solutions a_3: tip wash station a_4: stroboscope camera (b) stroboscopic image of dispenser tip for spot projection control b_1: dispenser tip stroboscope image b_2: droplet alignment scale b_3: dye solution droplet (c) schematic representation of piezo actuated dispensing tip principle

1.2.2. Veeco Dimension V (Atomic Force Microscope)

The Dimension V scanning probe microscope utilizes atomic force microscopy (AFM) and scanning tunneling microscopy (STM) techniques to measure surface characteristics for semiconductor wafers, lithography masks, magnetic media, CDs/DVDs, biomaterials, optics, and other samples up to 200 mm in diameter. Its laser spot alignment system and the ability to change scanning techniques without tools offer flexibility, ease of use, and high product throughput. The system's Hybrid X-Y-Z scanner provides six times lower Z sensor noise, precise X-Y control for manipulation of molecules, and highly accurate three-axis closed-loop control for "pulling" techniques. The AFM was used to investigate the polymer and dyed spot surface micro topography on a micro area.

1.2.3. Fluorescence microscope

A 'Axioplan 2 Imaging^{TM}' fluorescence and dark-field microscope was used for the optical characterization of the chemochip spots and other general microscopic estimations. The microscope is featured with dark-field imaging for the rough

topographic investigation. Also the UV excitation source enables to observe the quality and spotting success of the plotter. Almost all the dye spots were visible with characteristic colored emission wavelengths under UV excitation. The attached CCD colored camera can record the image on the operating computer of the microscope.

1.2.4. Reflection profilometer

A non-contact laser profilometer manufactured by UBM Inc. (now Solaris Inc.) was used for the thickness measurements of the DLC spots. The machine operates on the auto-focusing principle. The sample table is moved in the X-Y direction in order to scan the sample surface. The reflected light is measured as a function of focus adjustment and with the help of an analogue circuit the surface profile is created. It was more useful in case of DLC top layer spots, as they have relatively larger topography which is in the range of few tens micro meters. The machine has the maximum lateral resolution of about 1 µm which was quite sufficient to measure the DLC top and bottom layer thickness.

1.3. Developed fluorimetric chip characterization instrument

1.3.1. Sensovation opto-fluidic set up

A specially designed optical set up for chemochip fluidic experiments was developed. The resulting setup realized by the Sensovation AG (Stockhak/D) is shown in figure 2 a. It is in principle an optical bench equipped with following main part:

1) Halogen lamp as excitation light source
2) shutter for time limited light exposure
3) excitation-emission filter wheels
4) light propagation mirrors
5) CCD camera

Apart from these components some additional modifications were made in the optical setup. A ruler was set in front of the camera lens to mount the fluidic cell. This cell allows the passage of analyte liquid over the chemochip. X-Y-Z fine adjustment screws were present on the ruler to focus the fluidic cell or interaction micro chamber in front of the camera lens. (This interaction micro chamber, IMC is described further in section)

Typical component of microfluidic peripherals integrated with the optical setup are shown in the figure 2 b. The PTFE tube with 0.5 mm inner diameter was used for all fluidic connections. Syringe pumps, PTFE tubing, interaction micro chamber (IMC) constitutes the main parts of whole microfluidic peripherals. The halogen light source was used for visible range with a possibility of intensity variation. The snap of light was realized with the shutter gate situated in front of the light source. The gate has reproducible minimum exposure time from 30 ms. The images were created using the operation of this shutter. The short minimum time of few milliseconds provides the option of rapid imaging with fast scanning mode of the setup. The filter wheels were used to bring the appropriate filter set at excitation as well as emission position. The excitation filter wheel was set in front of the lamp shutter and emission filter wheel was set in between camera lens and camera as shown in the figure 2 b.

The light path way was adjusted by concave mirror to project the light on the IMC in a homogeneous manner. The mirrors also enable the excitation from the side of the IMC at an angle of 45°. The light beam diameter was about 2.5 cm to cover almost the whole IMC. The camera has intrinsic resolution of 2180 × 1472 pixels. This can be adjusted by selecting appropriate CCD binning area (number of CCD pixels in single read out) to realize rapid imaging. The camera creates a 16 bit image in tagged Image file format to have maximum data archive. The images were grey scaled with maximum value of 65536 per pixel. The grey scale value (GSV) of the image pixel is the function of fluorescence intensity. The image integration time was also adjusted according to the experimental requirements or fluorescence quantum yield of the spotted dye. It was then fitted inside the maximum pixel value to keep the pixel value below the maximum cutoff of 16 bit capacity covering the entire experiment for instance.

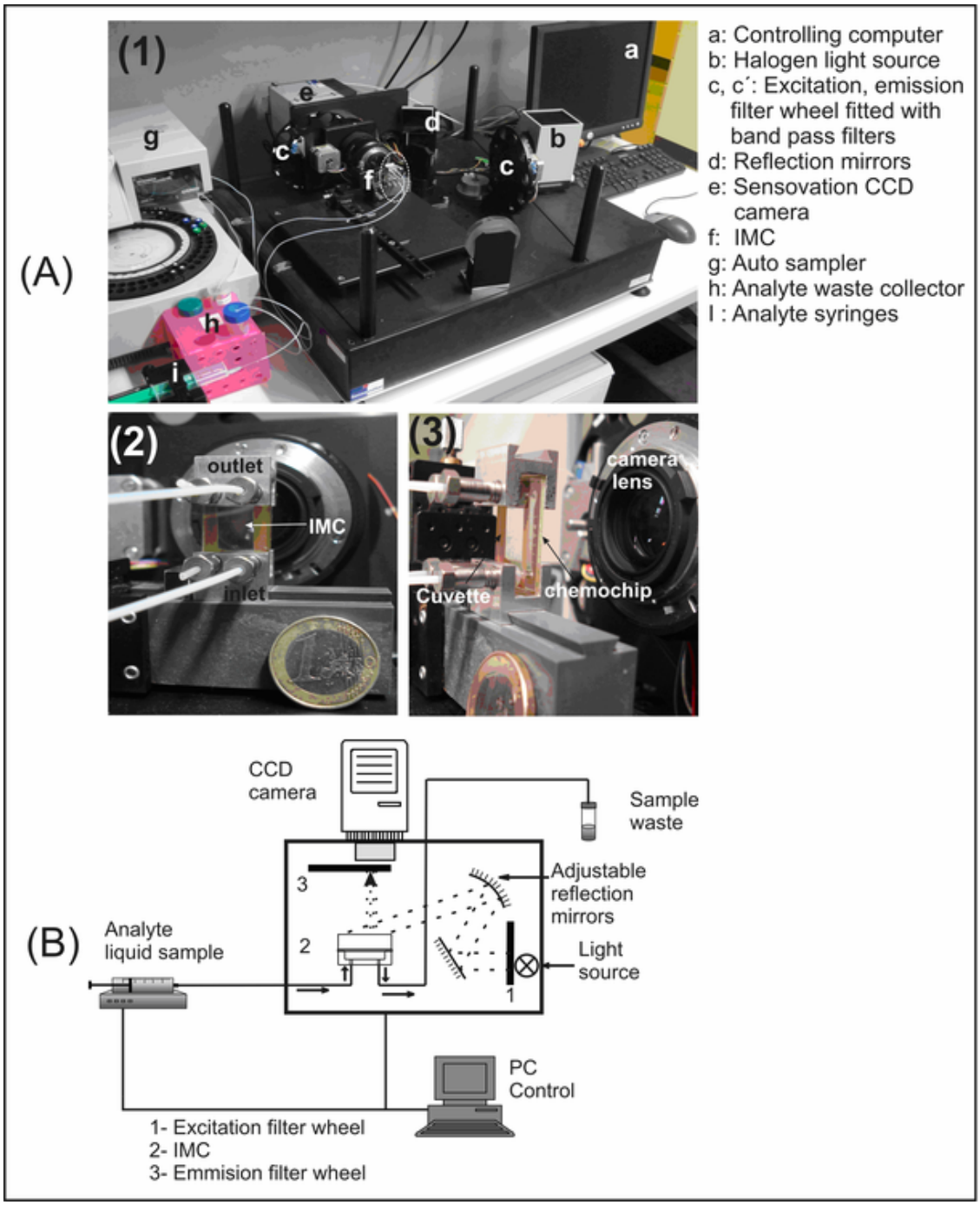


Figure 2: (A) The optical set up integrated with the high sensitive CCD camera and microfluidic peripherals. 1: An over view of the whole optical system and its main parts involved in the optical microfluidic chemochips experiments 2: IMC with fluidic inlet and out let for the liquid analytes mounted in front of the camera lens 3: Assembly of micro-cuvette and chemochip (B) Schematic representation of Sensovation optical set up with microfluidic peripherals

Table 2: Excitation and emission band pass filter sets used in the Sensovation optical setup.

	Filter Set 1	Filter Set 2	Filter Set 3
Excitation / nm	405	480	560
Emission / nm	430	530	640

All the function including camera parameters were automated using LabView^{TM} software application. All the external equipments like autosampler and syringe pumps were interfaced with the main setup. The experimental step sequence was fed in to the software and it was carried out automatically by the controlling computer.

1.4. Image Processing / analyzing

Software Image-J (free ware) was used for the image processing. A visually homogeneous area inside the single spot was chosen for calculating the average Gray Scale Value. The images were analysed for change in fluorescence intensity taking the spot intensity under water as reference and percentage change form reference intensity was determined. Image subtraction of the 'analyte image' (under the analyte liquid) from the 'reference image' (under distilled water used as a reference liquid) and vice versa was carried out to visualize spots changed in intensity. By decrease or increase in fluorescence the subtraction results revealed in the spot patterns showing only the spots which were changed in fluorescence intensity. Before obtaining the final spot pattern a number of 1000 was subtracted form all images to eliminate noise and artefacts which lies under this value.

2. Microfluidic arrangement for ChemoChip analytical experiments

2.1. The Interaction micro chamber (IMC)

The glass chip with dimensions of 25×15×1 mm^3 which is of the same as the polymer coated chip, bears a PTFE film which was adhered to one side with an engraved micro channel having a depth of ~0.06 mm (Figure 3) through which the analyte liquid was circulated for interaction with the spot array. The surface of the PTFE film was in direct contact with the swollen polymer surface concurrently acting as a gasket to seal the fluidic channels. These two chips were clamped together with screws. This compact liquid interaction micro cuvette (IMC) with the inlet and outlet apertures was the space for the analyte liquid and the micro spot array to interact with each other. Analytical experiments were carried out with the micro fluidic setup integrated into an optical bench, which mainly included syringe pumps, the auto sampler and the IMC (Figure 3). Distilled water was used as a reference as well as a washing liquid for the IMC. Delivery of the liquid was made inside the IMC by using syringe pumps under controlled delivery parameters of flow rate and sample volume. PTFE tubing with a 0.5 mm inner diameter was used to connect all fluidic peripherals.

The space for the interaction between prepared chemochip and analyte liquid is named as interaction micro chamber or IMC. It is made by cutting out a channel on the coated PTFE film on the glass or PMMA chip with inlet and out let apertures. The illustration of IMC has been made in figure 3. The PTFE layer acts as distance element containing structures for fluid conductance. The glass or PMMA cover slides were drilled with apertures for the inlet and outlet for the analyte fluid. Thus the formed micro chamber has a height of 10 µm with a total internal volume is 15 µL. It can be reduced or increased easily to a certain extent by changing the thickness and the shape of the distance element between the glass slides.

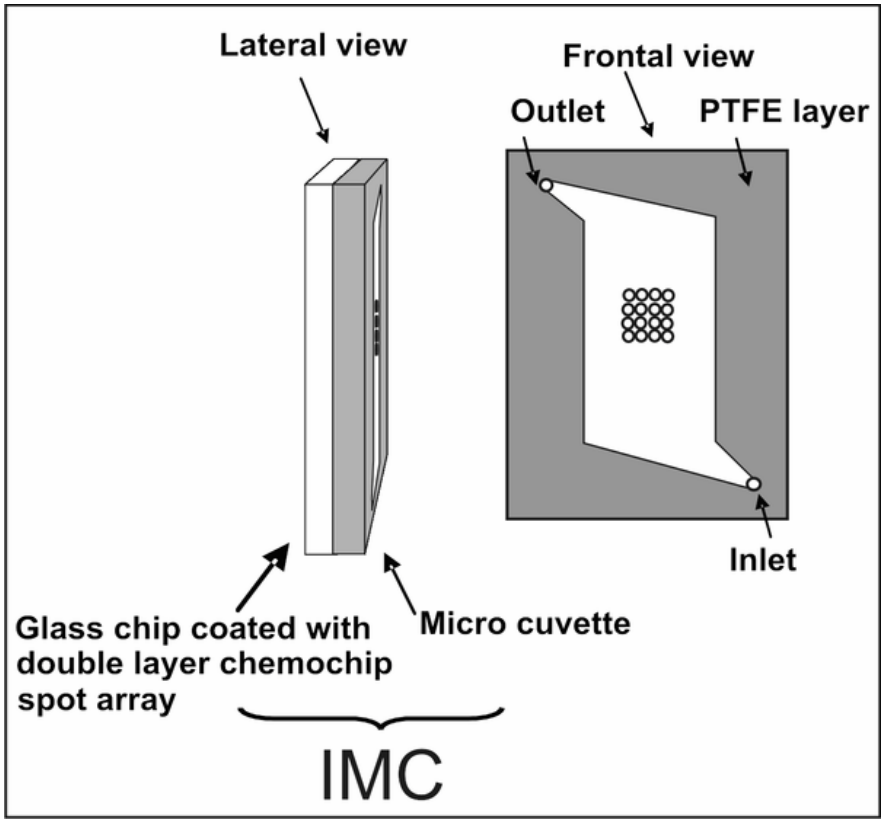


Figure 3: Interaction micro chamber (IMC) formed by overlapping of the polymer coated chip with an integrated micro spot array and the PTFE micro channeled cuvette.

3. Hydro-gel film preparation for single layered ChemoChips

3.1. Polyethylenglycol (PEG) films for single layer chemochips (SLC)

A polyethyleneglycol-bisacrylate based hydrogel thin film on a glass carrier was used as support for immobilization of the dye composition library. The film was prepared by photo-polymerization of a prepolymer mixture containing bisacryloyl-polyethyleneglycol (molecular weight 1900 Da), monoacryloyl-polyethyleneglycol (molecular weight 1000 Da) in water (50 % v/v). These methacrylic groups are incorporated into the polymer network during the polymerization process and provide a covalent binding of the polymer matrix to the glass substrate. The polymer has OH-groups as the immobilization interface between the polymer matrix and the fluorescent

dyes. The ethylenglycol chain provides a flexible spacer for the limited mobility to the immobilized molecules inside the swollen hydrogel matrix. Density of the hydroxyl groups was adjusted to about 20 mmol l^{-1} inside the swollen gel matrix. Swelling of the hydrogel matrix has been investigated for organic and aqueous solvents. The hydrogel thin film exhibits uniform and reversible swelling properties in water and organic solvents by a factor of two of the volume. This allows storage and processing of non solvated films without changes in their swelling capabilities. The polymer shear stress, induced by swelling, limits the preparation of stable films to a thickness up to 50 µm. In thicker films swelling induced forces either results in disruption of the gel matrix or in rupture of the gel from the surface of the glass carrier.

3.1.1. Basic polyvinyl alcohol (PVA) hydro-gel films for SLC

10 ml PVA solution of 7% (w/v) concentration in distilled deionized water was prepared in a stopper bottle by heating, till a clear solution was obtained. An aliquot of 500 µl of this solution was diluted to 3.5% (w/v) in a 1.5 ml Eppendorf cup by adding 500 µl deionized distilled water. To this solution 25 µl 5% (v/v) glutardialdehyde solution in water was added as a cross-linker for the PVA matrix. Then the solution was allowed to stand for 1 hr in order to bring out partial cross-linking of PVA and this leads to a semi hydrogel formation. The partial cross-linking helps in even distribution of polymer material on the activated hydrophobic glass surface. For activation, microscope glass slides were treated with activation liquid of tetraethylorthosilicate 5% (v/v) in ethanol solvent along with 1% (v/v) acetic acid and 1% (v/v) distilled water each. The glass slides were dipped in this solution for 30 min at about 60°C and cut in to 15 mm × 25 mm size chips. Tesa™ films were coated on these pre-activated glass chips. A square of 14 mm × 24 mm was cut out and a mould was prepared. 50 µl of the above mentioned semi hydrogel PVA solution was poured inside this mould and distributed on the entire chip evenly till the whole chip surface was wetted and covered with the solution. Every time a batch of 20 chips was prepared in this way and then allowed to be dry in ambient conditions till a homogeneous wrinkle free polymer film was formed.

3.1.2. Primary 4×4 micro spot array preparation on PEG films

For the basic array preparation four fluorescent dyes were used. These are mainly polarity and pH sensitive solvatochromic dyes, namely (1) H110 (2) N678 (3) Fluorescein sodium salt and (4) Rhodamine-B (Particularly for these dyes coupling reagent strategy was tried out with PEG films)

All the dyes were dissolved in the solvent dimethylformamide (DMF) and 20mM solution were prepared. These dyes were bound covalently to a polymer using *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodimide hydrochloride (EDCI) as coupling reagent, which forms an active ester linkage using the carboxylic acid group of the dye molecule and the hydroxyl group from the polymer itself. 4-Di(methylamino)pyridine (DMAP) and N-ethyldiisopropylamine solution (DIPEA) were used as catalyst and base respectively.

3.2. Extended large micro spot array preparation on PEG/PVA films

In this type of array the immobilization was done with out coupling agents. All the dyes were mixed in combinatorial form and spotted using Nano-Plotter. 32 binary fluorescent probes (doubly redundant i.e. 64) mixtures were prepared by mixing the stock solutions in equimolar quantities. The redundant spots were used to demonstrate the reproducibility of the array response. Resulting array map of the mixed dyes is depicted in figure 4.

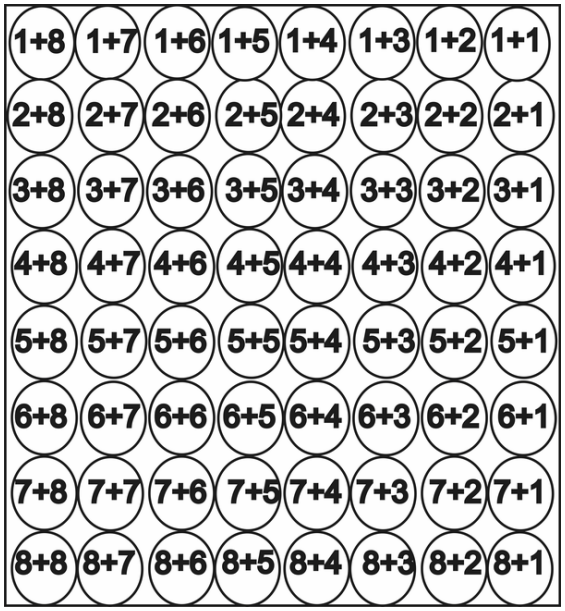


Figure 4: Map of 64 micro spots produced from eight different dyes, by binary combination. In square mirror fashion with pure dye on its diagonal from top right (1 + 1) to bottom left (8 + 8). The numbers are designated according to the dyes shown in table 2.3.

All 32 dye solutions were loaded into a 96 well micro-titreplate and placed onto the Nano-Plotter (Figure. 1a). Here, the array was prepared by spotting of about 4 nL solution onto the hydrogel film. Dispensed droplets formed spots of about 100 µm diameter in a precise and reproducible arrangement. The spotting process was monitored by using a stroboscope imaging (Figure 1b) camera integrated in the plotting device. The spotting pattern shown in figure 4 was created on the polymer chip.

After dye plotting, the chips were heated to 125° C for 7 minutes in hot air flow oven, in order to carry out immobilization reaction. We suppose that coupling reactions between hydroxyl groups of polymer and the dye or increasing cross linking leads to immobilization of dyes. Subsequent washing steps[1] with DMF, water / DMF mixtures and finally with distilled water were give till constant spot fluorescence intensity was obtained. In order to avoid any pH influence caused by the polymer matrix the chip was washed with phosphate buffer of pH about 7.0 ± 0.5 before use and the arrays were dried under vacuum.

[1] *All the washings were given with the flowing solvent conditions. Each time 1ml of solvent was passed dropwise over the chip with the help of micropipette.*

3.3. Hydro-gel film preparation Double layer chemochips (DLC)

3.3.1. Bottom layer (indicator layer) film preparation

Bottom layer or so called indicator layer was prepared from hydrogel PVA matrix itself. Stock solution bottom layer estimated for 5 chips batch was prepared in a 1.5 ml Eppendorf cup as follows:

7% (w/v) PVA solution in water 500 µl + 1 mM fluorescein sodium salt 50 µl +

5% (v/v) in water glutardialdehyde 50 µl + 1 M HCl 1 drop

50 µl of this solution was spread on the framed[2] activated glass chip.

The chips were dried in the ambient temperature and then cured at 125° C for a short period of 10 sec. These chips were then washed with water and buffer of pH 7 alternating cycle for couple of time in order to wash out the remaining acidic residues of used HCl and unbound fluorescein in the film formation process. Chips were again dried in ambient conditions in a partially covered Petri dish.

3.3.2. Top layer (separation layer) film preparation

The PVA top layer was prepared in two different ways: **(a)** with whole film modification and **(b)** with local film modification (array format)

Two different modifiers were tested for the top layer: Sucrose (whole film modifier), glutardialdehyde (local film modifier)

3.3.3. Top layer whole film modification with sucrose

Three types 1ml stock solution of 7% (w/v) PVA were taken: 1) with dissolved 0.03 gm/ml sucrose 2) 0.003 gm/ml sucrose and 3) the pure PVA solution which was used without any modifier as the third type of top layer.

Combination of volumes and concentration of sucrose content of above stock solutions were used on the framed, activated glass chips to prepare the top layer. In this way films with varying thickness was realized. 6 different types of chips were prepared with sucrose as modifier and varying the top layer thickness. These DLCs are summarized in following table 1 and table 2.

[2] *As per described in section 2.3.3 (b)*

Table 3: Stock solutions used to prepare the DLC top layer thickness modification

PVA stock solution concentration (w/v)	Sucrose content gm/ml	Total volume of 7% PVA used /µL	Top layer thickness variation using constant 0.03 gm/ml of sucrose
7%	nil	100	15 µm
7%	nil	200	20 µm
7%	nil	300	25 µm
7%	nil	300	25 µm
7%	0.03	300	25 µm
7%	0.003	300	25 µm

3.3.4. Top layer local area film modification (array format)

In this type of film the top PVA layer was modified with glutardialdehyde. The initial pure film using 300µL of 7% (w/v) uncross-linked PVA was formed on the chip. Various concentrations of glutardialdehyde cross-linker solution were spotted on preformed top PVA layer with the help of Nano-Plotter. This formed the locally cross-linked spot arrays. After spotting, the chips were cured shortly for about 30 s at 125° C on a hot plate. Then they were washed in immersed condition for 10 min in a buffer solution of pH 8 in a closed Petri dish on a magnetic stirrer. This washing was carried out until the uncross-linked PVA top layer film was pilled out from the surface due to continuous stirring. The chips were cleaned carefully with tissue paper to remove any of the remaining uncross-linked PVA from the top layer and then washed with deionized distilled water thoroughly and dried in air.

Table 4: Stock solutions used to prepare the DLC local area top layer modification

Glutardialdehyde concentration in water (v/v)	Num. of droplets ×100pL
1%	200
4%	200
8%	200
12%	200

4. Chemicals and Materials

4.1. Indicator dye for micro spot array on single layer hydrogel films

The fluorescent indicator dyes are the main building blocks of the chemochips spot array. Ten dyes of various excitation and emission wavelengths have been used. The fluorescein and its derivative dyes e.g. dye 1, 4, 6, 7 from table 4 were mainly pH responsive, where as the other like dye No. 2, 3, 7[3], 8 are responding to the polarity change in the micro environment. The source and other technical details of the used dyes have been summarized in the following table.

Table 5: Fluorescent indicator dyes[3] used for the preparation of the SLC and DLC

Dye No.	Name (commercial name and source)	Dye No.	Name (commercial name and source)
1	H110, Molecular Probes Inc. Leiden, The Netherlands, CAS- 73024-80-3 (Abs: 497 , Em: 519)	6	Eosin Y, Merck KgaA. Darmstadt, germany. CAS- 17372-87-1 (Abs: 552 , Em: 610)
2	N678,Molecular Probes Inc. Leiden, The Netherlands, CAS- 96801-39-7 (Abs: 467 , Em: 536)	6[4]	Prodan Merck KgaA. Darmstadt, germany CAS-70504-01-4 (Abs: 361,Em: 498)
3	N316, Molecular Probes Inc. Leiden, The Netherlands, CAS- 88235-25-0 (Abs: 467 , Em: 539)	7	Eosin B, Neolabs GmbH. Heidelberg, Germany CAS- 548-24-3 (Abs: 361, Em: 498)
4	Fluoresceine (sodium salt) Fluka Chemie GmbH. Switzerland. CAS- 518-47-8 (Abs: 490 , Em: 520)	7[5]	Nile red Neolabs GmbH. Heidelberg, Germany CAS-7385-67-3 (Abs: 552, Em: 663)
5	ETH 4003, Fluka Chemie GmbH. Switzerland. CAS- 192190-91-3 (Abs: 550, Em: 630)	8	Rhodamine B Merck KgaA. Darmstadt, germany. CAS- 81-88-9 (Abs: 540, Em: 590)

4.2. Other material and chemical used

Bisacryl-polyethylene glycol (M.W. 1900 Da) Roth GmbH, monoacryl-polyethylene glycol (M.W. 1900 Da) polyvinyl alcohol (M.W. 16000 Da) Acros

[3] *See Appendix 1 for dye structural formula*
[4] *Dyes tried out for immobilization shown in figure 3.4a*
[5] *Dyes tried out for immobilization shown in figure 3.4a*

chemicals Ltd., glutardialdehyde (Merk, GmbH). The selected polymer exhibits hydro-gel properties in cross-linked form. The swelling of polymer when in contact with water, helps the diffusive interaction of analytes with the indicator molecules. *N*-Ethyl-*N*′-(3-dimethylaminopropyl), carbodimide hydrochloride (EDCI), 4-di(methylamino)pyridine (DMAP) and N-ethyldiisopropylamine solution (DIPEA) were used as coupling reagents to bind with polymer matrix in one of the types of single layer chemochips. The general solvents used were ethanol, toluene, hexanol, dimethylformamide, methanol all solvent used were of A.R. grade from Merck GmbH.

5. Micro spot array characterization

The spotted arrays for chemochip applications are mainly composed of general indicator dyes. These array spots are prepared on polymer surfaces in sub-nano liter volumes; it sets the first task to characterize these spots, their formation process and physical properties. As mentioned previously, there has not been much investigation done on micro spot physical characterization as an array. The spotting process is a key step in the preparation of the spot microarray. The preparation of array was followed by its surface characterization for the quality of spots and immobilization of dissolved materials. Conventionally the ellipsometric technique has been used for DNA array characterization along with AFM where high resolution imaging is required.

5.1. Array spot formation and surface characterization basic concept

In general, when a droplet of a volume about 100 pL hits a polymer surface, number of factors play role and influence the spot formation process. The actual spot formation process lasts from about 1 s to 5 - 6 min depending up on these factors. Of course the obvious parameter is the number of droplets (volume) used to create the spot. But keeping this factor constant, the most important components of the whole processes are; the used solvents, and secondly the dissolved material in it. The vapor pressure of

the solvent determines the speed of evaporation. In addition, evaporation rate is influenced by phyico-chemical properties like polarity, ion strength, and dissolved substances, which also decide the wetting behavior on the polymer surface. Figure 5.a illustrates the spot formation process of a pure solvent without any dissolved material.

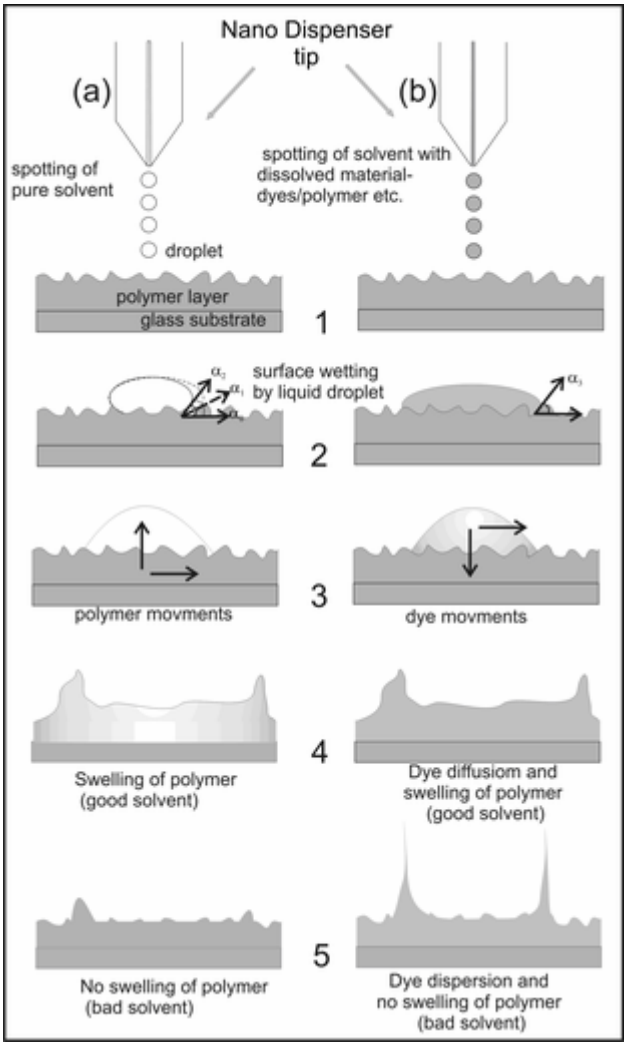


Figure 5: Possible surface alterations and physical processes taking place during the solvent drying process after the spotting by the Nano-Plotter

The second step is the formation of the drop on the polymer surface. Different solvent like DMF and pure deionized water has different contact angle with the PVA surface therefore, resulting in to difference in wetting behavior. This will also be influenced by the additional dissolved substances in the solvent. Therefore this will change the final spot diameter (step 2 figure 5. a, b). The polymer swells to varying

degrees depending up on the solvent properties, immediately after the drop formation starts (by multiple droplets from the dispenser tip, Step 1 figure 5. a, b).

The polymer material can be partially dissolved in the solvent droplet, swelling itself in the solvent medium as indicated by the up arrow in the step 3 in the figure 5.a and then towards the periphery of the spot during solvent evaporation process indicated by the horizontal arrow in the same step. This leads to heightening of spot area shown in step 4. On the other hand if the used solvent is a bad solvent for the polymer then the solvent can not penetrate the matrix to greater extent and only spread on the polymer surface leaving a less significant spot topography as per shown in the step 5 in figure 5.a but a larger spot diameter. This process could be a deciding factor for the final spot height and the outer rim formation. These processes can be altered according the dissolved material in solvent during the spot formation. Apart from the contact angle variation (step 2 figure 5.b) the interactions between the dissolved material and the polymer matrix are added in the whole process. Along with the solvent if the dissolved material like dyes or other polymers are well soluble in the base polymer matrix then the process is similar to that of step 3 in the figure 5.a and it takes place with more dissolved material.

The difference could be the transport of dissolved material in to the base polymer matrix and whole of this mass towards the spot rim. In the other case, if the base polymer matrix is not a good solvation medium for the dissolved substances then material remains at the surface after spotting and gets transported to the outer periphery along with the evaporating solvents. This gives high concentration of these materials on the spot boundary leading to a strong and sharp spot rim as shown in step 5 of figure 5.b. In this case it does not play role in the spot height and it is decided only by the base matrix solubility in to the incoming solvent and the solubility of the dissolved dye or polymer in the base polymer matrix.

5.2. Optical characterization of micro spots using light microscopy

In order to investigate basic solvent polymer matrix interactions, some potential spotting solvents and a fluorescein dye as a model substance were used by a standard spotting procedure. The spot features have been investigated using fluorescence optical microscope, also equipped with dark-field illumination objective. The spots compositions depicted in figure 6. are given in table 6. For all the spots the base polymer matrices used were spin-off PVA and PMMA films at about 2500 rpm.

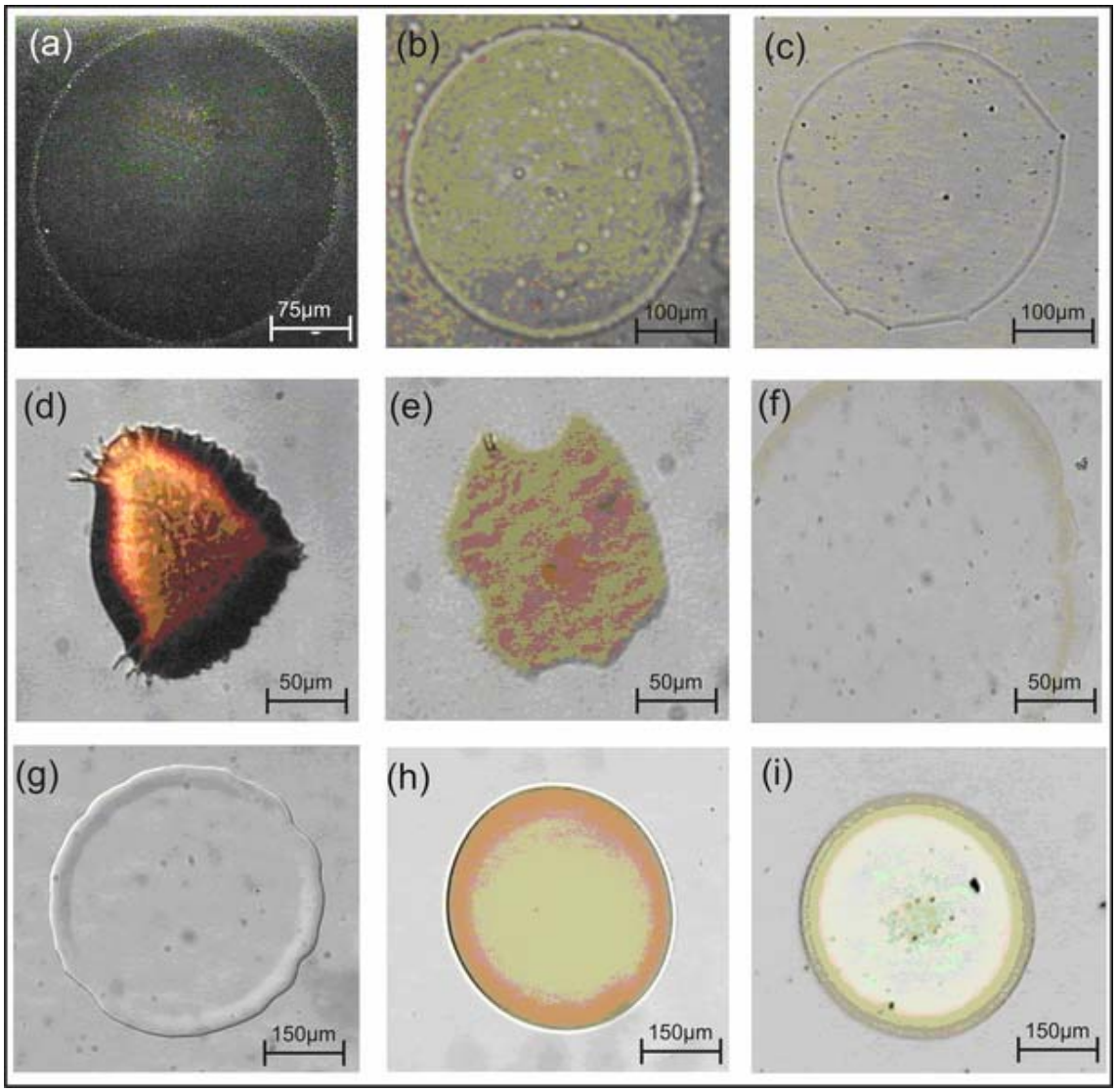


Figure 6: Microscope optical spot characterization prepared from using water and DMF as solvent on different polymer surfaces. The spot composition is described in table 5.

Table 6: Spot composition details depicted images in figure 6, 200 droplets/spots were used of 100 pL droplet volume. PVA concentration used for spinning 7% w/v in water and PMMA concentration was 10% w/v in toluene.

Image	Spot composition	Base polymer film
(a)	Water spot	PVA
(b)	DMF spotted on PVA	PVA
(c)	Toluene spotted on PMMA	PMMA
(d)	Fluorescein in water on PMMA	PMMA
(e)	Fluorescein in DMF on PMMA	PMMA
(f)	Fluorescein in DMF on PVA	PVA
(g)	PVA in water	PVA
(h)	PVA mixed with Fluorescein	PVA
(i)	PVA mixed with Fluorescein	PVA

The formation of micro spots varies according to the physico-chemical properties and the interaction between the spot components and surface. Figure 6.a. shows that pure water spot[6] on the PVA coated surface and 6.b shows the DMF spot on PVA surface respectively. Here it can be seen that water spot has less diameter as compared with the DMF spot. And water spot seems similar to the toluene spot prepared on PMMA surface. Image (d) and (e) are the spots prepared with water and DMF respectively on PMMA film with dissolved fluorescein. A clear distinction can be made in the spot size. In case of water as the solvent, the spot with dissolved fluorescein contracts due the hydrophobic interaction with PMMA surface. The dissolved fluorescein dye is also hydrophilic hence the spot agglomeration takes place. In case of DMF as solvent due it's less polarity than water it wets the PMMA surface more and spot size changes. The images (g), (h) are the spots of PVA (pure) and dye respectively on spin off PVA film. There is no much difference between the spots with and without fluorescein. Therefore it can be seen that the spot physical properties are controlled by the higher molecular weight PVA. Also the dye is seen more homogeneously distributed

[6] *The light micrograph of the spot prepared with pure water has very poor contrast with bottom PVA film and can not be seen through the normal light microscope mode. Therefore the image has been obtained under dark-field mode of the same microscope shown in figure 6a*

in these types of spots. Image (f) is the fluorescence image of the same type of the spot taken under UV excitation. This image demonstrates the optical intensity distribution of the spots.

The fluorescence optical microscopic images of various micro spots can be investigated for the dye distribution and optical homogeneity of the spot. In figure 7, spots prepared with varying composition of the water and DMF mixture with constant concentration of 0.15 mM of dissolved fluorescein have been shown. The amount of DMF is increased from image (a) to image (f) as per described in table 6. In this case the polarity of the solvent is essentially varied for each spot. Also, it is observed that the homogeneity of the dye over the entire spot area is different as per the solvent polar character. The dye distribution is most homogeneous for pure DMF and pure water. There is inner rim formation for the spots prepared with the mixed solvents. The spots are imaged at completely dried conditions; therefore the effect of solvent on fluorescence intensity is absent at this point. And the fluorescence properties are mainly controlled by matrix effect. It can not be estimated at this stage whether the PVA matrix-dye interaction has increasing or decreasing effect on fluorescence intensity. One can not investigate the dye material transport in vertical direction with the help of these fluorescence images. This characteristic transport (distribution) of dye and change in intensity needs to be investigated.

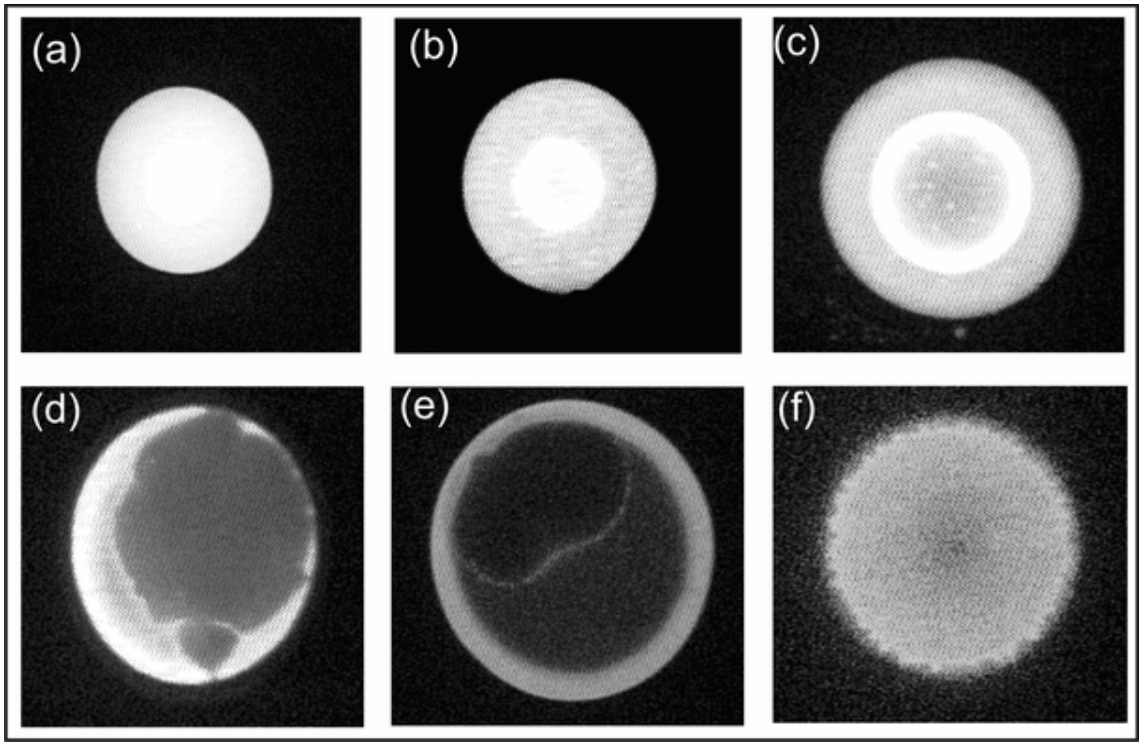


Figure 7: Microscope optical spot characterization in fluorescence mode prepared in varying concentration of DMF in water as solvent with constant concentration of dissolved fluorescein. Images a - f: fluorescence mode images.

Table 7: Solvent compositions used for the spots in figure 7 the spots were prepared using 100 droplets/spots with 0.15 mM fluorescein dissolved concentration in each spot

Imges	solvent composition / %	
	DMF	water
a	0	100
b	5	95
c	35	65
d	55	45
e	75	25
f	100	0

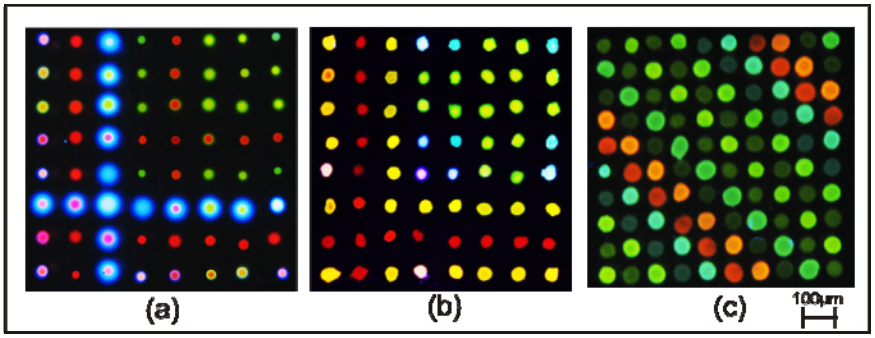


Figure 8: Fluorescence microscope image with UV excitation showing the micro spotted array for eight different dyes and its distribution over the PVA surface. The array is prepared using a 20 droplet/spot (a) Binary mixed dyes shown in table 2.3 sec. 2.2.1 in DMF *with* predissolved coupling reagents. (b) Binary mixed dyes shown in table **4** in DMF *without* pre-dissolved coupling reagents. (c) Dye spot prepared with mixing of host material like crown-ethers.

Spotted mixed and pure dye arrays on the PEG films are imaged under UV excitation. The images are depicted in figure 8 (a) contains dye (1-7, table 6) and their mixed combinations as per shown in figure 8.a along with the coupling reagents. The image (b) contains dyes (1-8, table 4) spotted without coupling reagents. In image (a) a clear separation of two dyes is seen due to their differential transport in to the base polymer matrix during the spot formation process. This separation is almost absent in case of the dye mixtures spotted with out coupling reagents. Image (c) is the array containing dyes (1-5 table 4) with mixed model host molecules like crown ethers. The spotting is carried out as per spot map diagram shown in figure 4. The UV image shows the spot which contains crown ethers are increased in fluorescence intensity. So there is no strong rim effect in case of spot arrays (b) and (c) and also the dye separation due to the differential dye transport in the base polymer matrix is absent.

6. ChemoChips hydro gel micro spot arrays for characterization of common solvents and complex multi component liquids

6.1. Primary micro spot array for pH and polarity characterization:

The development of fluorescent spot arrays for chemical sensor application requires knowledge about the influence of solvent polarity on the fluorescence behavior of each sensor spot. In order to optimize each dye spot in the microarray, we have investigated the solvatochromic fluorescence behavior of different spot types simultaneously, under the influence of series of solvent mixtures with varying polarity (Fig.10). The numbered E_N^T empirical polarity unit has been used for the classification of polarity of the analyte samples discussed here. Hence, the determination of a set of general chemical properties can be applied together with selected indicator functions. Polyethylene glycol films coated on glass (IPHT, Jena) have been used as an immobilization substrate. The elementary dyed micro spot array was applied for the characterization of the fluorescence behavior of single dye spot in response to the basic physico-chemical properties of the analyte liquid like polarity and pH. A basic 16 spot microarray (Fig. 12) was subjected to solvent mixture and pH buffers using the microfluidic setup. The array was prepared such that a number of such arrays would fit in a single microfluidic cuvette as shown in figure 11 to test signal reproducibility in array response. The smaller arrows inside the channel represent the multidirectional flow of the analyte liquids.

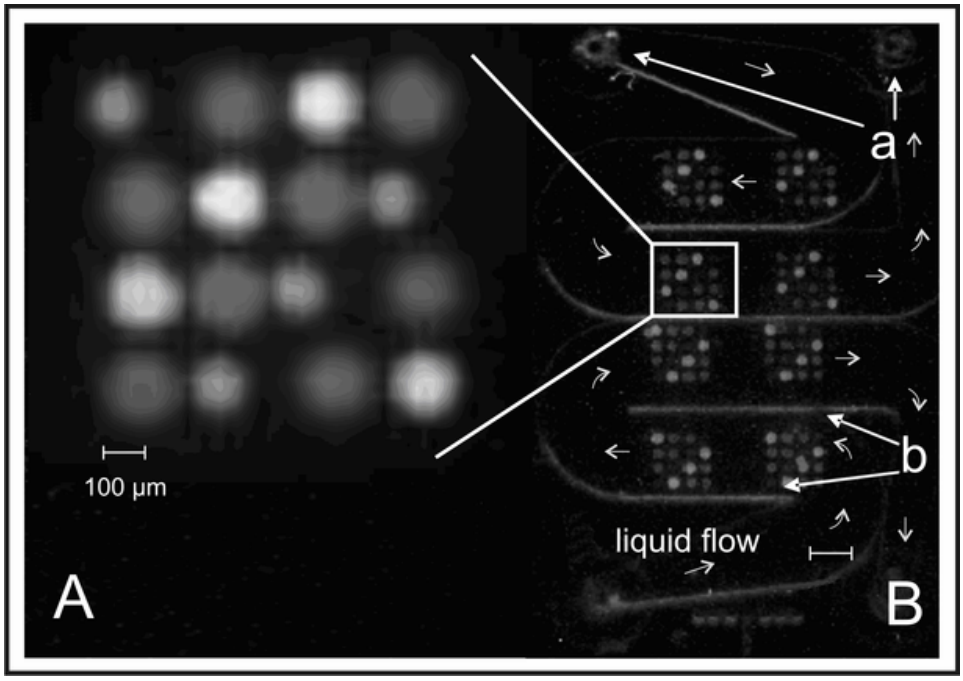


Figure 9: A. Magnified CCD image of an array taken with the Sensovation optical setup. B. Dyed micro spot array inside glass cuvette micro channel. a. Inlet and outlet apertures. b. Glass cuvette with micro channel edges. The liquid flow inside the cuvette is represented by the small arrows in the channels.

The single SLC bearing a series of identical micro spot array was covered with the micro cuvette for the liquid passage over the chip. The array was fitted exactly in the cuvette channel as per shown in figure 11 and the micro channel edges acted concurrently as the gasket on the swollen polymer film which sealed the channels for fluidic passage.

6.2. Analytes

Dimethyl sulphoxide (DMSO) was diluted with varying amounts of water in order to prepare the analyte sample with different polarities (Table 7). Different standard buffer solutions were prepared and used as pH analyte samples (Table 8).

Table 7: Polarity samples used with E_N^T empirical scale values calculated from the absorption λ_{max} measurements of Reichardt's betaine dye

DSMO content in water % (v/v)	100	90	70	50	30	10
E_N^T Scale	0.44	0.59	0.81	0.92	0.96	0.99

Table 8: pH solutions used as analyte probes

Buffer used	Citrate-Phosphate	Citrate	Citrate	Phosphate	Phosphate	$NaHCO_3$
pH	4.7	5.3	6.0	7.4	8.0	10.0

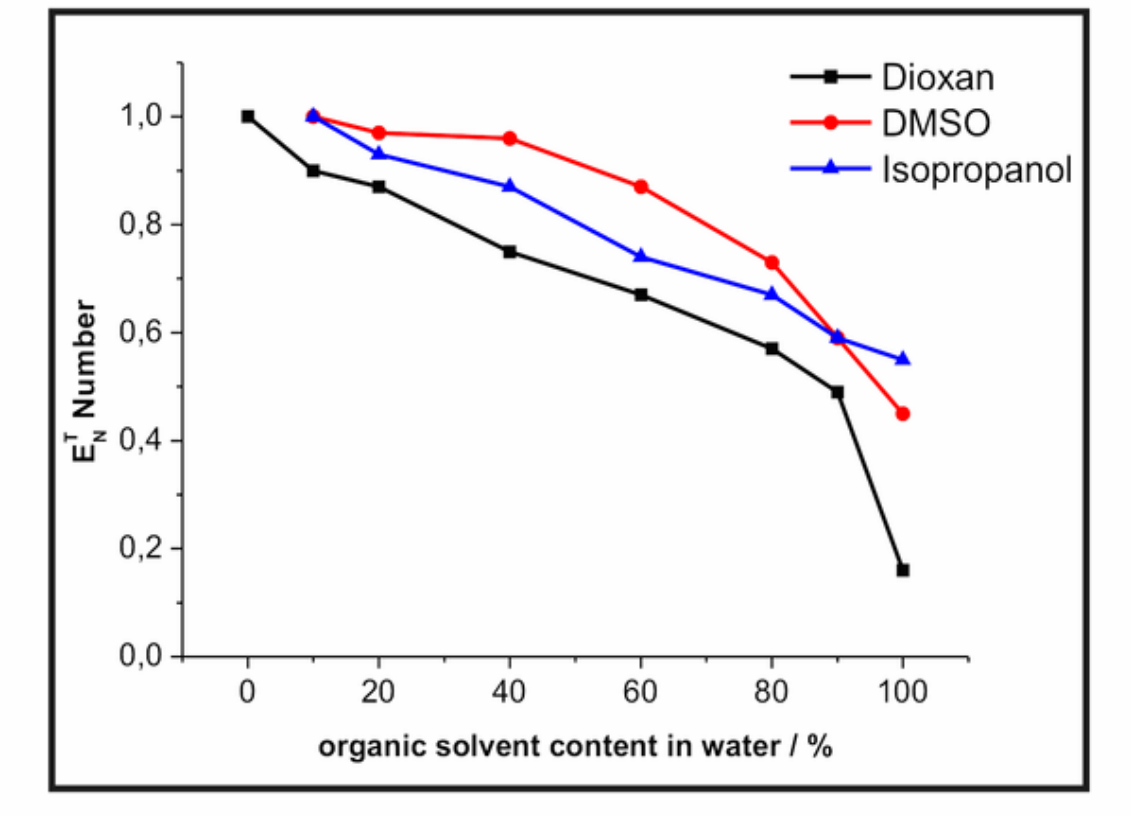


Figure 10: Polarity of the analyte sample expressed in E_N^T number empirical units as a function of organic solvent content in water. The values are calculated from absorption λ_{max} measurements of Reichardt's betaine dye.

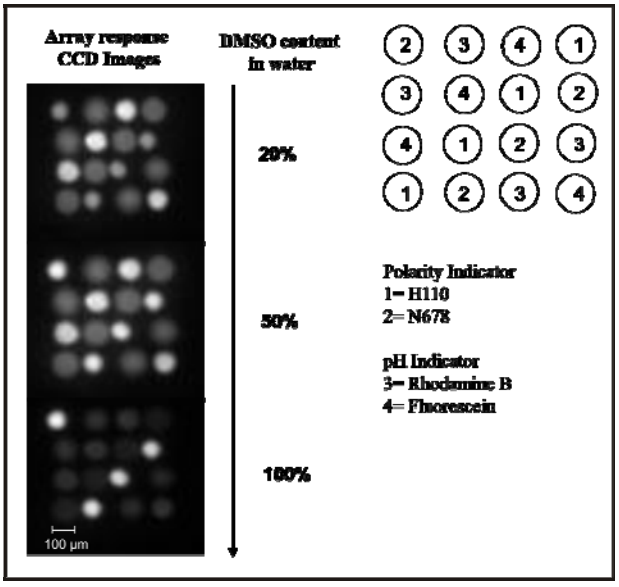


Figure 11: Optical response of a micro spot array towards the polarity of analyte liquids. Fluorescence intensity of different dye spots changed characteristically as the polarity of the analyte liquid.

The first array pattern response of the 4 × 4 array towards the polarity change in the applied liquid analytes is shown in the figure 10. A spot pattern with varying intensity is generated due to the contrasting response of the different dyes towards the different analytes. The dye N678 dye goes up in the intensity and rest of the dyes decrease in intensity. The varying response and the redundant distribution of the spots in side an array leads to the generation of a spot pattern which can be further useful for quick analyte identification. A detail intensity characterization is discussed in the following section.

6.3. Liquid analyte characterization

The digital images recorded with the CCD camera were saved in the TIFF format and analyzed for the GSV of the spots. These images had an upper cut off at GSV 6553 and below 3000 (only in this type of experiments) were treated as the back ground count rate with an estimated noise of 100. The absolute GSV count of the dye spot varies with the amount of dye bound to the polymer surface and duration of the

exposure to the excitation light. Each dye spot has its characteristic initial intensity which depends on its emission intensity at the given excitation wavelength and its spectral overlap with the excitation and emission band pass filters. According to the type of dye used in the construction of the spot array, the initial GSV of the images differ slightly from array to array and spot to spot depending on the reproducibility of the preparation procedure steps of the array.

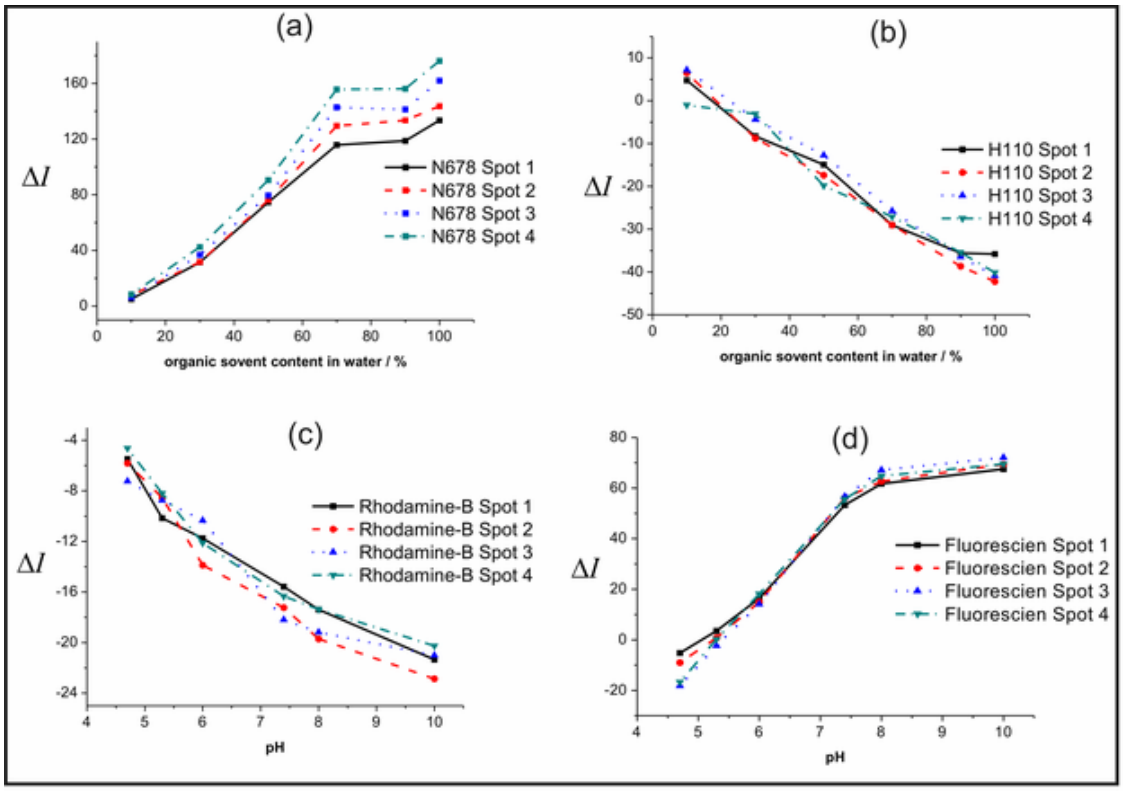


Figure 12: Reproducibility of the fluorescence signal from different spots of same composition depending on polarity and pH, after spot fluorescence intensities were analyzed for percentage change with respect to reference intensity in the solvent (distilled water). a - b percentage fluorescence intensity change of the polarity indicator dyes spots. c - d percentage intensity change of pH indicator dye spots.

Single spot intensity analysis was made to characterize the analyte properties and their gradual changes. ΔI was used for the representation of the fluorescence intensity change taking place in the spot dependent on the analyte properties. Equation 1 was used for the ΔI calculations.

$$\Delta I = \frac{I_{analyte} - I_{ref}}{I_{ref}} \cdot 100 \qquad \text{Eq. 1}$$

ΔI = percentage change in fluorescence intensity

$I_{analyte}$ = fluorescence intensity after analyte interaction

I_{ref} = fluorescence intensity of reference image

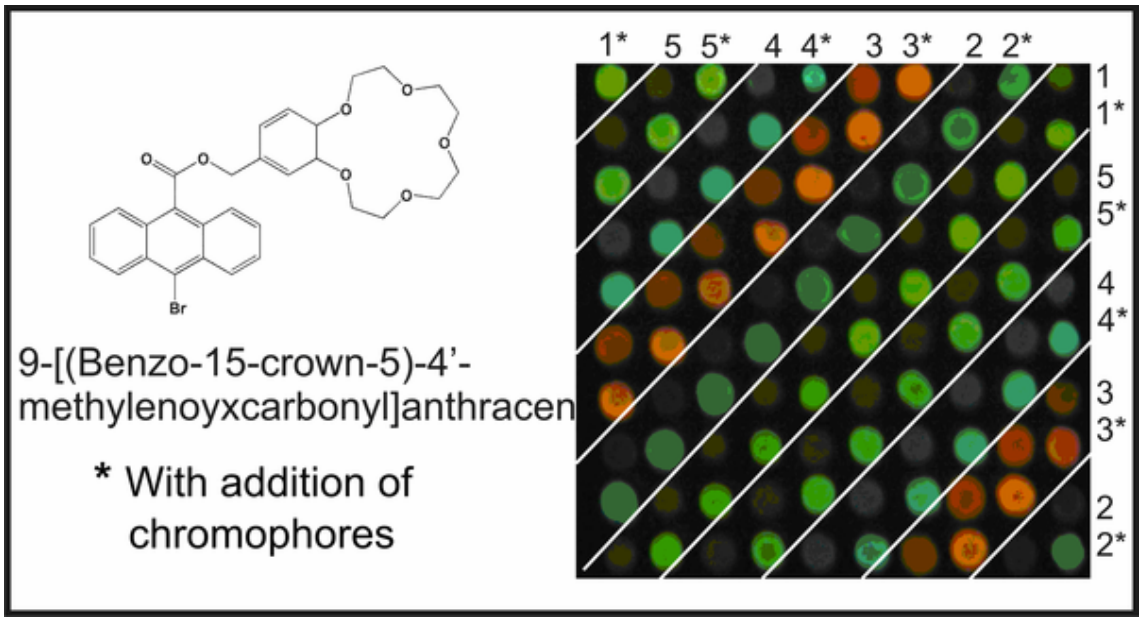
Generally, the gray scale or color values are added or subtracted to know the change in the optical signal which is always based on the reference picture. Eq.1. This signal was plotted against the solvent composition (Fig. 12). As mentioned earlier, there might be leaching of dye observed in the case of some analytes. In order to have a better calibrated system we treated the new GSV of the thoroughly washed spot in the reference image as the zero value (reference value) and calculated the percentage change in fluorescence intensity from that new value. The array showed a typical pattern of fluorescence alteration with respect to the change in the analyte sample characteristics. A polarity response is represented in figure 12 (a) and (b). Dye 1 and dye 2 (dye designation according to figure 11), which were typical polarity indicators showed opposite behavior. Spots of dye 1, positioned along the diagonal of the array showed a gradual decrease in fluorescence intensity with the decrease in polarity of the sample analyte. On the other hand the fluorescence intensities from the spots of dye 2 were increased with decreasing analyte sample polarity. A similar response was also shown by dye 3 and dye 4, but the change in fluorescence intensity was comparatively lower. The extent of fluorescence intensity change between dye spots 1 and 2 is also different. Dye 2, is more sensitive towards change in polarity in comparison with dye 1 and shows a percentage increase in fluorescence intensity of up to 180%. On the other hand the percentage decrease in fluorescence intensity of dye 1 goes down only by about 45%.

The other two (pH indicator) dyes used in microarrays were Rhodamine-B (dye 3) and Fluorescein (dye 4) in their salt forms. The sodium salt of Fluorescein forms an anionic dye, while the chloride of Rhodamine-B makes it a cationic dye. These dyes once again show opposite behavior towards the pH analyte liquids. (See Fig. 12 c, d)

Fluorescein is a pH indicator fluorescent dye which shows a pH dependent ionic equilibrium also in dianionic form with high fluorescence intensity. Hence it also shows a considerably larger percentage fluorescence change which is up to 95% while with Rhodamine-B the percentage intensity change lowers to around 22%. This data also emphasized the fact that although the GSV of each individual spot was scattered considerably, the percentage change in the fluorescence intensity of each spot was in good agreement with the exception of dye 1, for lower polarity analyte samples; however the deviation is systematic.

6.4. Micro spot arrays with mixed crown-ethers for liquid analyte characterization

The initial UV characterization revealed that the arrays showed good homogeneity of the spot in optical characterization. There was no dye and mixed chromophore separation observed in the matrix due to differential lateral transport of dye material in the polymer matrix. The spots with mixed crown ether showed an enhanced fluorescent intensity as shown the spot lines with * in figure 13. These arrays were prepared in order to check the influence of crown-ethers on the detection ability of the original indicator dyes. In the UV characterization, the mixed spots showed no emission wavelength shift (color change), however enhancement of fluorescent intensity was observed.



1= N678	2= N316	3= Rh. B	4= H110	5= Fluorescein
1*= N678*	2*= N316*	3*= Rh. B*	4*= H110*	5*= Fluorescein*

Figure 13: SLC micro spot array prepared with inclusion of fluorescent chromophore (crown ethers) as host like molecule along with the indicator dyes. The spot were prepared by spotting the mixture of the equimolar (20 mM) solution of the two species. The figure 4 describes the map of the spot array. Using 20 droplets/spot (200 nL/droplet). The image is taken under UV excitation at 230 nm. The inset table shows the used dyes and the spot with marked spots are included with crown ethers.

This altered intensity may not give special detection ability to the indicator spot but can be used as an additional variable as the grey scale intensity vector of the 16 bit image. Therefore these arrays were subjected to the analyte characterization. The standard solvent analytes were introduced on the chip and the spot pattern generated for the distilled water was taken as the reference patter to compare with the spot patterns generated due to other solvents. The dye numbers referenced in following discussion are referred according to the figure 13.

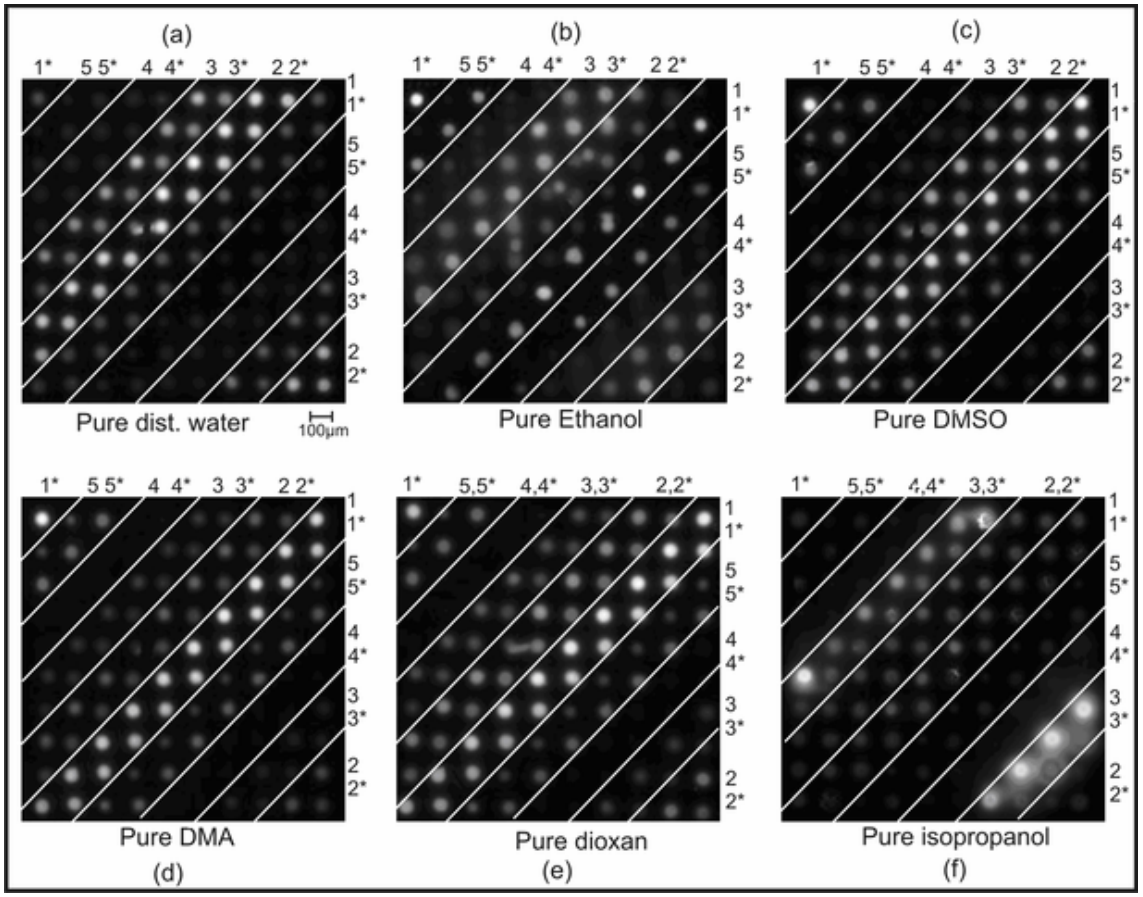


Figure 14: Micro spot array pattern created by the spotted dye array with mixed crown ether host molecules. The arrays were prepared in a redundant manner according to spot map shown in figure 4. The images of the array were recorded under the analyte and shown as it is without any further image processing. The observed spots are the spots with altered intensity after analyte interaction with reference to the dist. water image. The dark areas in the image are spots which were decreased in intensity from its original value.

The above mentioned arrays were characterized using the solvent of varying polarity and proton strengths. In figure 14, all six solvent analytes showed distinct spot patterns with varying spot intensity for each group of spots. For water which is the highest polar protic solvents of the lot; dye groups (1,1*), (2,2*), (3,3*) were increased in intensity. It can be seen that the two types of spots of the same indicator dye shows similar intensity under water as analyte. However, under ethanol the spot pairs belonging to the same group differ significantly in their intensity. The spot group 1,1* for example showed a significant rise in intensity for the mixed dye spot (dye 1*) as

compared with its counter part in the same group (dye 1). In case of isopropanol which is a slight higher molecular weight alcohol and slightly less polar than ethanol showed a different spot pattern than pure ethanol due to dissimilar optical behavior of the spot pairs of the group for isopropanol, especially the groups (1,1*) (3,3*) (5,5*) . The visible spot groups differ in case of the two alcohols. This renders the array a different spot pattern easily recognizable from the pure ethanol spot pattern. The other solvent analytes DMA; DMSO, dioxan which come under a same category of polar aprotic solvents, show a similar type of spot pattern. But in these spot patterns there is an appearance of spot group (5,5*) along with the other spot group as the compared with the polar protic solvents. The spot group 5,5* is prepared from the fluorescein dye which typically shows response towards the proton strength of the analyte. Although, the spot pattern is same for these analytes, they bear different fluorescence intensity and have been compared in the further discussion.

The polar aprotic solvents like DMSO, DMF, and DMA are compared in figure 15. The polarity of the DMSO is supposed to be highest in the lot. DMF would have lower polarity and DMA the lowest among the three solvents. The fluorescent spot response towards these solvent is compared taking an example of a dye spot from groups (1,1*) and (2,2*) from each image as indicated in the figure 15. A simple ratio of the absolute intensity of the selected spot pair from the respective groups shows the difference in the spot signal. This gives a possibility to distinguish between the analytes with similar spot pattern but with varying spot intensity by comparing their intensity vector. The ratio of the selected spot of group (1,1*) and (2,2*) is lowest for the DMSO and highest for the DMA, whereas the intermediate value of the ratio was obtained for the DMF. The trend in the increase of the ratio is in agreement with the analyte polarity. As the ratio value increases (1.18, 1.85, 3.00) with decrease in analyte polarity, the ratio value for an outstanding strong polar protic solvent of the given class like water is much lower (0.34) than the aprotic solvents.

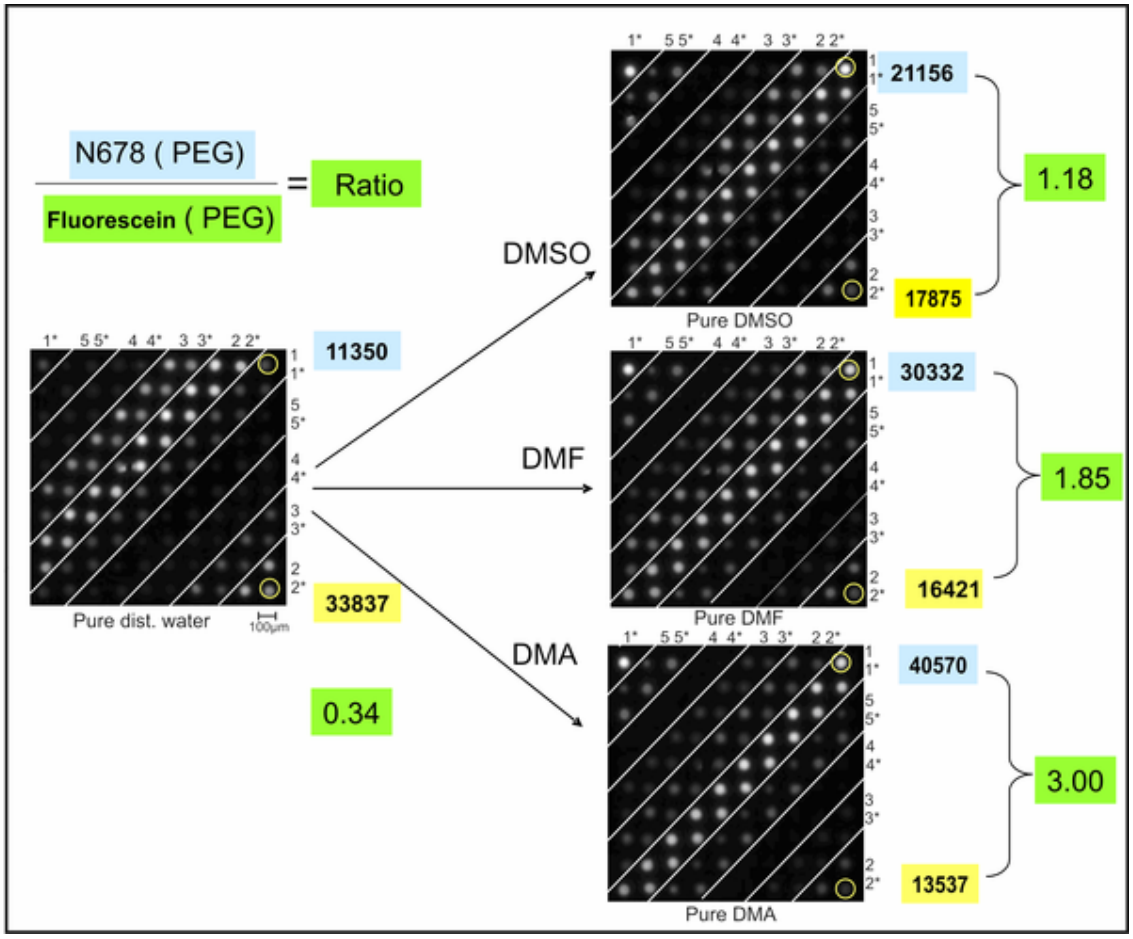


Figure 15: The fluorescence intensity of the individual spots has been compared for a same class of analyte solvents with varying polarity.

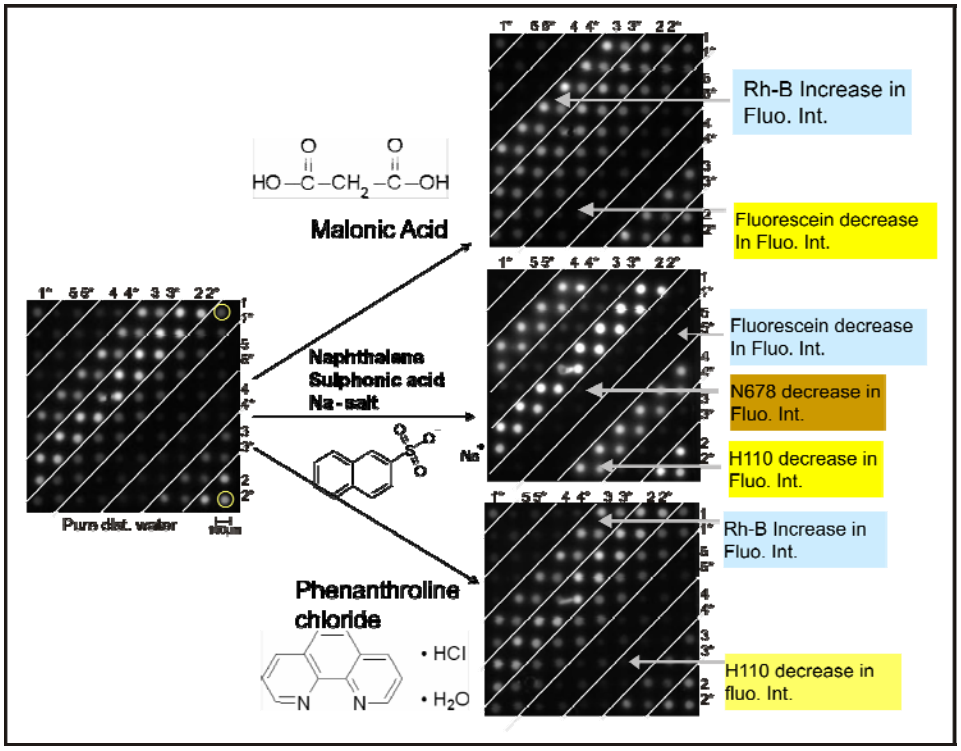


Figure 16: Comparison of aqueous analyte solutions with varying acidity and chemical nature. (The analyte concentration used in the water was 0.5 M)

The aqueous solutions of different acidic species showed a different spot pattern. In order to differentiate the variation in the spot pattern and fluorescence intensity of these aqueous solutions, the water image was taken for the reference and the variation in optical signal was compared for some dye spot groups. Malonic acid which is water soluble aliphatic acid shows a spot pattern as shown in the figure 16. Rhodamin-B (3,3*) showed increase in fluorescence intensity as compared with the intensity under water. The spot groups (1,1*), (2,2*) also showed increase in intensity towards malonic acid solution. Whereas the other two groups (4, 4*) and (5,5*) which are fluorescein derivatives showed a complete diminished fluorescence as its standard response towards the low pH solutions as seen in the earlier results in figure 14. For naphthalene sulphonic acid and phenanthroline hydrochloride which are slightly higher in their pH values made N678 and H110 i.e. (4,4*), (1,1*) groups to rise in intensity. But dye group (3,3*) which was intensified for malonic acid showed fluorescence decrease for naphthalene sulphonic acid but was increased once again in case of phenanthroline hydrochloride. Thus, the variation in fluorescence intensity is due to the complex influence factors which are playing role at the recognition site (array spot) in the form

of a network. These interactions should be considered here in order to understand the spot optical behavior.

6.5. Interaction network binary dye spots

The complex interaction factors may play important role in the optical response of the spots prepared with the binary mixed dyes. An account therefore has been taken of the spot response and number of influencing factors. We have opted for fluorescent dyes in the visible light range. The selected dyes are known to be responsive towards pH- and polarity changes (87-90). But, the molecular fluorescence phenomenon being a very sensitive one, and can be affected by some more parameters other than just pH and polarity. The high sensitivity of the fluorescent dyes has been used as a powerful sensor tool by analyzing the data output from the fluorescent micro spot array.

The depth of the acquired images contains detailed information about the analyte interaction measured on the gauge of grey scale values. We have adopted two approaches to arrive fingerprinting criteria. The first is an individual spot intensity analysis inside the array which gives the single spot response to the analyte interaction. The spot intensity curves are used for the simultaneous characterization of binary mixtures of two analyte liquids. The second approach is the image-processing and data handling which gives the distinguishing fluorescence spot patterns of the micro spot array.

The fluorescence phenomenon is influenced by various parameters like temperature, viscosity, pressure, hydrogen bonds, ionic strength, quenchers, pH and polarity (91). In the case of micro spot array made of polymer embedded fluorescent dye mixtures, these factors play a key role in the fluorescence alteration. Considering this fact, the fluorescence response of an individual spot reports about the micro environment change of the fluorescent species and the changes in the interaction network (Figure 17). If different analytes influence on the network with different interaction types a spot specific response can be expected. In this way the individual spot response as well as complete array pattern can be analyzed.

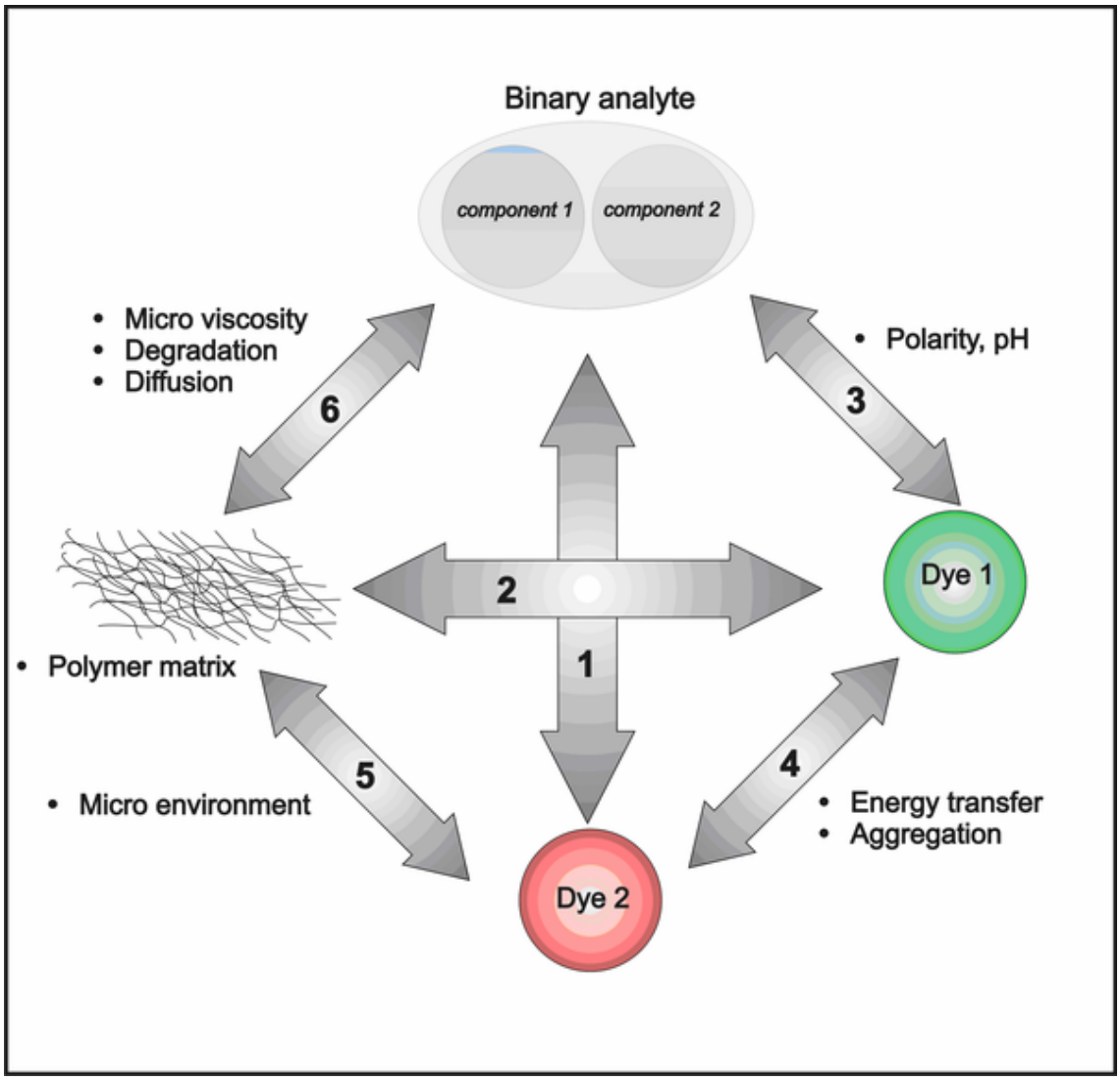


Figure 17: Proposed interaction network between various components of the single dye spot in its micro environment. Each numbered arrow represents a type of interaction affecting the fluorescence signal.

The interactions which constitutes the micro environment before introduction of analytes can be classified as ‘intrinsic’ (i.e. 2, 4, 5 Figure 17) because they are the integral part of the dyes - matrix interaction network. These interactions cause the array pattern of the reference image. After analyte application the interaction network parameters are changed and change in fluorescence intensities can be observed. Factors which change these reporter system can be called ‘extrinsic’ (i.e. arrows 1, 3, 6, Figure

17) as they are caused by the analyte. It was shown before, that different polymer environments affect the response behavior of the fluorescence intensity due to the local physico-chemical properties like polarity, hydrophobicity, matrix swelling capabilities etc (92, 93). The molecular proximity and local analyte concentration in the confined binary spot will influence the network in a different way. Gradually changed interactions like π - stacking- (94) H-bond or other energy transfer processes will cause a different fluorescence response. So, a complex response was expected in case of the binary dyed spots. For different mixed dye spot a different behavior to the same kind of analyte was expected.

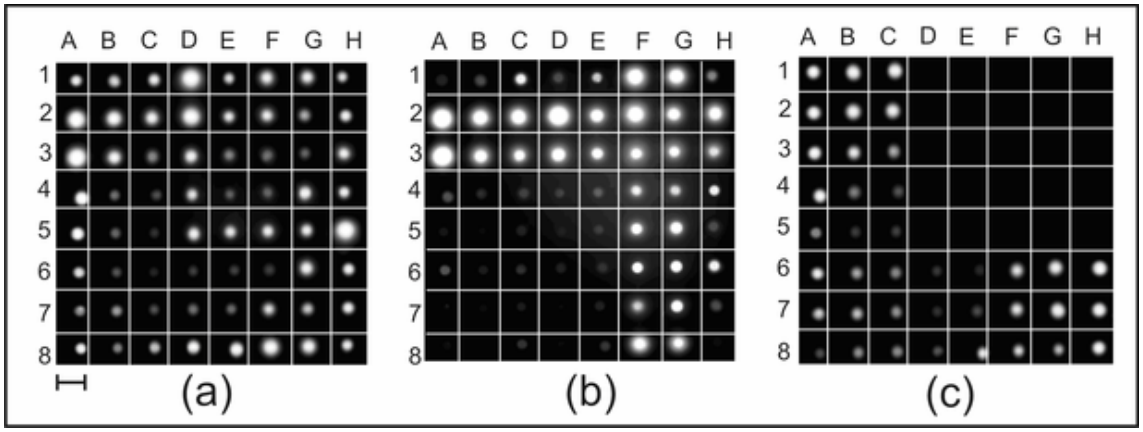


Figure 18: a: Gray scale CCD image of the micro spot array recorded in dry state using filter set 1 (Ex-405 nm, Em- 430 nm). b, c: The images of the same array under aqueous wetted conditions using filter sets 2 (Ex- 480 nm, Em- 530 nm) and filter sets 3 (Ex-560 nm Em- 640 nm). The scale bar under image (a) represents 300 µm.

Prepared arrays were characterized by recording images under UV excitation of 320 nm wavelength. A spot diameter of about 75-100 µm depending on the polymer surface wetting of the different dye solutions were realized. The positioning of the spots is mainly dependent on the conditions of droplet formation from dispenser tip of the plotter. Obtained grey scale images are shown in figure 18. All spots inside the array were detected if the appropriate excitation and emission filter sets were used (for filters see table 2, sec 3.3.1). As reference for all measurements the array was imaged in dry state after assembly of the IMC. The reference image was taken from the dry array to avoid any extrinsic influence on the interaction network. The swelling state of the

polymer matrix is an important factor for the interaction network. If analyte solutions are applied the swelling of the polymer is influenced. To record these interaction changes caused by the analyte the reference image was taken under dry conditions.

6.6. Relative spot intensity analysis

In order to characterize the individual spot response towards the gradual change in the interaction network, the spot intensities response was analyzed for the different water ethanol mixtures. Received spot intensities for some selected spots are shown in figure 19. Intensities were calculated as changes of the spot intensity related to the reference image intensity using eq. 1. The dye designations discussed in this section are according to the table 4, sec 6.1.

$$\Delta I = \frac{I_{analyte} - I_{ref}}{I_{ref}} \cdot 100 \qquad \text{Eq. 1}$$

The pure dye 1 containing spot (dye H110, table 2.1) shows decreasing fluorescence intensity with increasing ethanol content. The dye 3 (N316) containing spot shows a contrary behavior (Figure 19a). Both spots respond on the local polarity of their micro environment differently (95). The spots with the binary mixture of these two dyes show a superposition response of both single dyes. The total fluorescence intensity lies between the intensities of the spots of pure dyes. But, the behavior of dye 3 dominates the fluorescence response to the increasing ethanol content. A different behavior was found in case of the dye set 2+4 and 4+8 (Figure 19b, c). Here, the intensity curves of both pure dye spots lie at higher intensities than the mixed dye spots. However, the mixed dye spots show hybrid behavior compared to its pure dye spots. At low ethanol concentration the spot behaved like the pure dye 2 spot (0 - 5%). But, the mixed dye spots show an increasing intensity with medium ethanol content (15 – 55%). At higher ethanol content (75 – 100%) dye 4 dominates the fluorescence behavior and the spot intensity increases. The lowering of the entire fluorescence intensity and the hybrid response can be attributed to the gradually changed interaction network. In case

of dye combination 4 and 8 a complex behavior was observed as well (Figure 19c). The mixed dye spot intensities are quite higher compared with the single spot response.

6.7. Fingerprint spot pattern

For the identification of different liquids, the images were processed primarily to generate fingerprint spot patterns. This was achieved by subtracting the 'reference array image' from the 'analyte array image' and vice versa. The resulting image reveals pattern, which visualize the intensity changes of the individual spots after interaction with the analyte liquid. Figure 19 is an example for the received spot patterns for methanol.

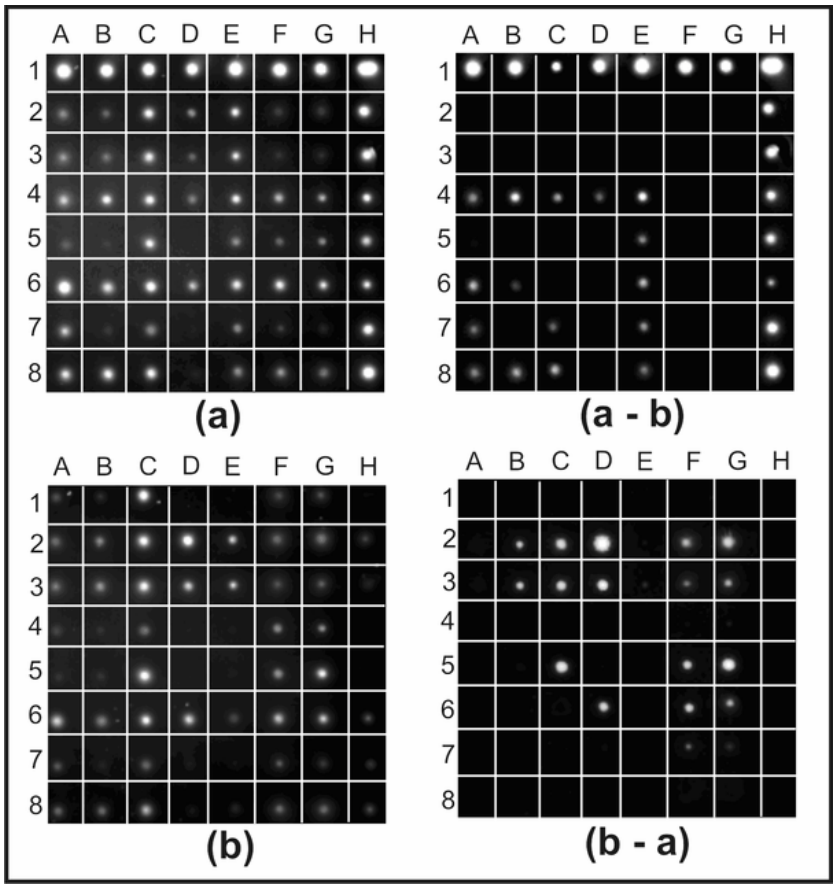


Figure 19: a: reference image (dry array) b: image under pure methanol (filter set 2 table 2 sec 3.3.2). Spot pattern obtained after image subtraction: a - b: fluorescence diminished spot pattern. b - a: fluorescence intensified spot pattern.

In figure 20 the pattern for the spot intensity changes are depicted. Both intensified and diminished spots were observed in the array response. Figure 20 (96) shows the diminished spots and figure 20 (b-a) the intensified spots. The examples of spot H1 (figure 20 image a) (pure dye 1, H110) and G2 (pure dye 2, N678) can be considered to see the distinguishing results for the image subtraction. As the pure dye 1 spot decreases in intensity from its original value, the analyte subtraction image (figure 23, image a - b) showed the intensified spot H1 response. Whereas dye 2 showed increase in intensity for the same analyte sample, therefore the spot G2 appeared in the subtraction image (figure 19, image b-a). The dark area in the resultant array of course shows the spots that remained unchanged. The redundancy of binary dye spots inside the array (mirror symmetry line from H1 to A8) is very helpful to recognize experimental artifacts like gas bubbles or solid impurities.

The earlier mentioned individual spot intensity change analysis and PCA analysis give additional information about the behavior of the individual spot with respect to the analyte liquid. But, if fast and direct analyte identification is concerned, the visualization and subsequent differentiation of array pattern images is of great advantage. The subtraction images of the array provide the best choice to visualize the pattern changes.

In figure 20 received response patterns for different alcohols and alcoholic liquids are shown. The comparison of different alcohols with decreasing polarity (ethanol, isopropanol, hexanol) shows comparable spot patterns with different intensities (Figure 20 a). Here, the spot pattern and intensity response is caused mainly by the solvent polarity. Only some spots show a non monotonous response. Different water ethanol mixtures show a totally different pattern. Here, the polarity of water dominates the response. But, the individual spot intensities can be used for the determination of ethanol content (Figure 20 b). All aqueous samples show a similar spot pattern. The spots in the left bottom corner of the array were present in all kinds of alcoholic mixtures in figure 20 b. But, with increasing water content the individual spot intensities decreases. Observed monotonous effects for the different alcohols and

different alcohol concentrations are predominantly caused by the sample polarity. Only some spots were found which do not show a monotonous behavior (shown earlier in figure 10).

The examination of more complex alcoholic samples like common alcoholic beverages resulted in to individual patterns for each beverage (Figure 20 c, d). Here, the physico-chemical sample properties and specific ingredients make influence on the interaction network in a more complex way. The resulting patterns are individual and were not dominated by the ethanol concentration. If the aqueous ethanol patterns (Figure 20 b) are compared with the beverages (Figure 20 c) we can clearly see the difference between beers and 5% aqueous ethanol for instance. More over the spot patterns even for Indian and German beers are different. The comparison of different wine types result patterns with a certain similarity but different from the aqueous ethanol pattern (dry white wine and sweet white wine).

Also examples of beverages with higher alcohol content like Vodka and Gartenmeister liquor show individual patterns. In group 124c an euqivolume mixture of Gartenmeister and white wine has been shown. The spot pattern shows the dominance of wine over the Gartenmeister as the left conrner spot appear in the resulting spot pattern. But spot H1 which is strong polarity indicator spot shows its appearance as the mixture would have lower polarity than the pure wine due to the addition high ethanol containing (69% v/v) Gartenmeister.

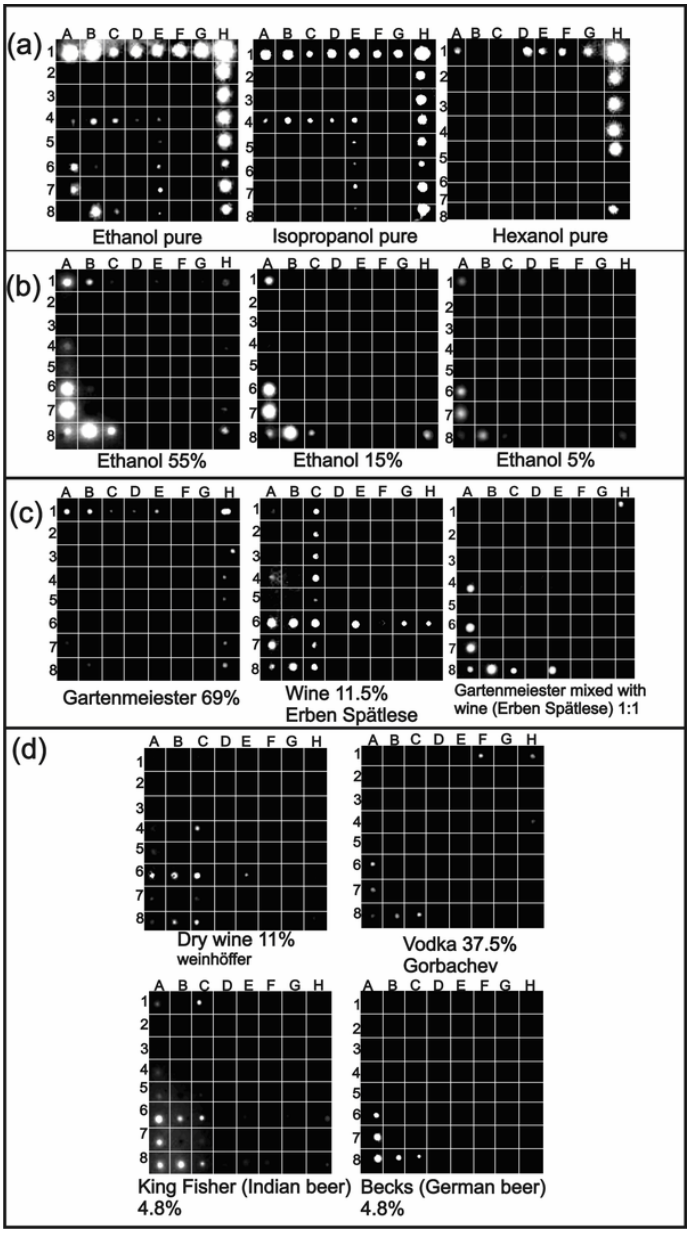


Figure 20: Fingerprint spot patterns revealed as reference subtraction images from various simple as well as complex composed alcohol containing liquids and common beverages. a: Alcohols with increasing aliphatic character. b: Aqueous ethanol mixtures. c: showing spot patterns for common alcoholic beverages like Gartenmeister and white wine with a spot pattern for their mixture in 1:1 volume ratio. d: some more array response for alcohol beverages are depicted. Ethanol concentration is shown in % vol. All the images are obtained by subtracting analyte image from reference image with filter set 2).

Observed individual patterns are an indication that the array responds to a plurality of factors and not only to the polarity or pH of the sample. For pattern recognition the criterion for grouping spot patterns is in principle based on the appearance of the spots.

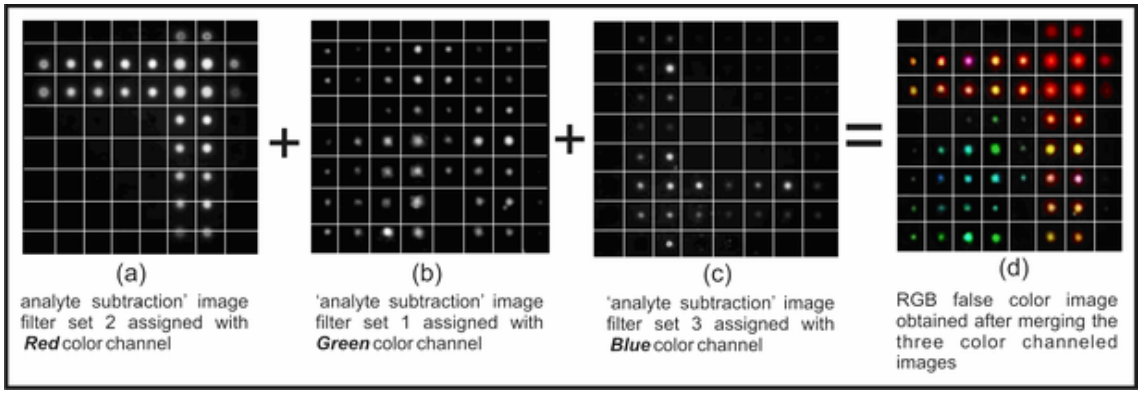


Figure 21: The spot patterns generated by the performing (b-a) type (analyte image subtracted from reference image) for Gartenmesiter are shown for the three filter sets. The images were assigned with colored channels as shown in the (a, b, c) images and were merged together to generate a false color image using 'Image-J' software.

So far the spot intensity in the array is a 32 dimensional vector (with 32 different fluorescent species). The selected images were assigned with RGB color channels. This makes the array response a 96 dimensional vector by applying 3 RGB values to the same spot from three different images taken at different wavelengths. Using the software Image-J the color channeled images were merged together to create a false color image with the spots having different RGB counts shown in figure 21 corresponding to the GSV in the original images. This gives a rigorous, multidimensional way for assigning fingerprinting spot patterns to the analytes.

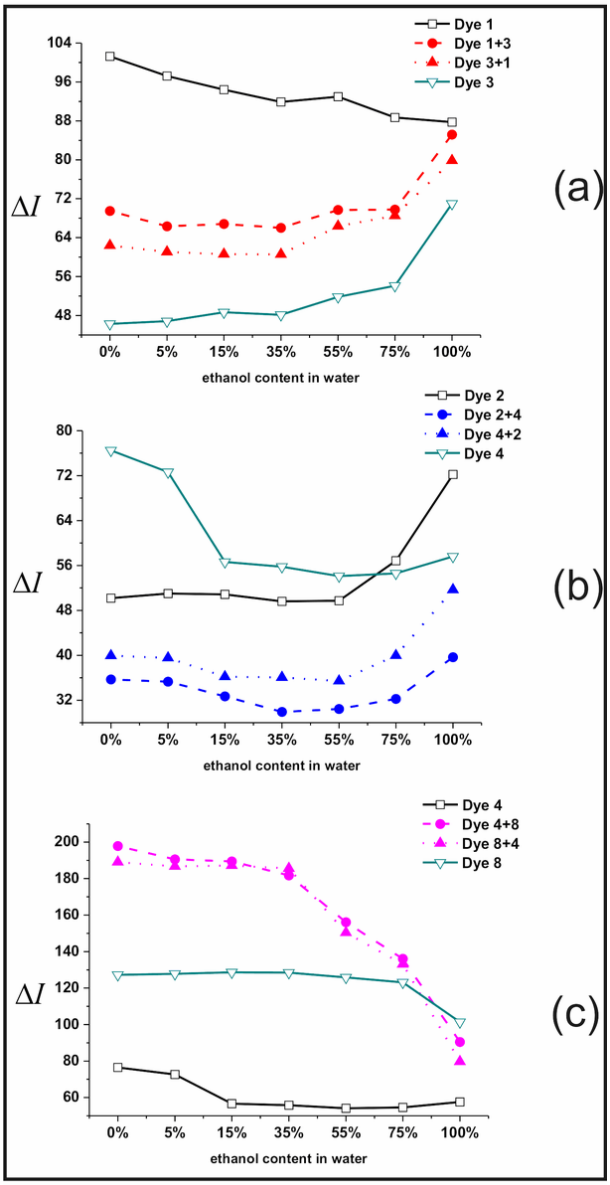


Figure 22: Individual spot intensity analysis of the pure dye spots and their binary mixtures as indicated on the graph legends. The images considered for this analysis are from the appropriate filter set with maximum spectral overlap with the pure dye spectra. Figure 4a - filter set 2, 4b - filter set 2, 4c - filter set 3 (table 2).

Furthermore, the binary combination shows an ethanol related response which can not be attributed to the behavior of a single dye spot. At ethanol content higher than 35% the intensity shows a steep increase. The mixing of the two dyes in the polymer matrix gives rise to a non-typical spot behavior. Moreover, dye 4 which was used for the second (figure 22 b) and third (figure 22 c) mixed spots is essentially the same

species but its influence on the interaction network is quite different. For the mixed dye spots always two redundant spots are shown in figure 22 a – c in order to demonstrate the quality of the array spot preparation and the rather reproducible response of the spots with same composition. The results demonstrate that the response of binary dye spots do not depend in a monotonous way from effecting analytes and are well suited for the preparation of array which responds with specific patterns for different applied analytes.

7. Simultaneous separation and detection of liquid analytes via transport modulator double layer chemochips (DLC)

The development of micro devices for chromatography (97-99) and for micro electrophoresis can be traced back to the earliest micro reaction activities (100-102). The miniaturized and chip technology-based chromatographs are always equipped with a separation unit extended in the substrate plane. Parallelization is only possible by increasing number of these separation structures. The possible degree of integration depends on the area needed by a single separation unit. This can not be significantly reduced if the separation takes place collateral to the substrate plane. As a result separation units should be working perpendicular to the substrate plane, which means in the normal direction of the chip is required. The realization of miniaturized separation units with perpendicular orientation would enable the development of highly integrated analytical micro devices. The principle of the double layer chemochip (DLC) is based on the stacking of the film functions. The splitting of functions of a single film into a film stack with specialized functional layers is derived from multilayer resist technology, where the inherent resist functions (resistivity, sensitivity against exposure, planarization of relief) are split onto two or three layers (103, 104). So the two different processes, the controlled transport of analyte and its recognition are fitted here in series. In DLC the control of diffusive analyte transport from the analyte solution to the recognition sites is localized in the top layer and the recognition and indication are localized in the

bottom layer (105). The bottom layer contains a fluorescence dye and its fluorescence activity is modulated by the analyte components. This influences the absorption or emission band (solvatochromic effect), the chemical state of indicator dye (chemical reactions like protonation / deprotonation, complexation) or the fluorescence quantum yield (energy transfer, quenching). Here, the immobilization of fluorescein as a pH-sensitive fluorescence indicator dye inside polyvinyl alcohol (PVA) films is chosen as a simple example of the indicator bottom layer.

The double layer micro spot principle can be presented as a simple possibility for combining the advantages of parallelization by chip array detection and the separation of chemical species by different transport rates in the thin swollen polymer films. Aqueous solution of malonic acid, phenanthroline hydrochloride and triethylamine were used as the model analytes to demonstrate this principle. The first two species which are acidic in natrure and are of different anion sizes and have varying interactions with the polymer matrix and the third which is an organic base; can be used to get the DLC response towards basic solutions, as a contrast with the first two analytes.

7.1. Development and application of DLCs with general top layer modification

Before steping in to more complex DLC spot array format, the initial development with the DLC was done with the modification of the entire top layer as illustrated in figure 23 (a, b) The top layer films were prepared in two ways, firstly by changing the used volume of polymer solution and secoundly with the predissovled polymer modifier in the polymer solution. A schematic working and readout principle has been illustrated in figure 23 (c). The (a) part of the figure shows the top layer modification by varying layer thickness. As the direction of interest is normal to the indicator layer, the time required for the dissolved analyte molecule to reach to the idicator layer surface varies. This variation in time ‘τ’ is measured exploiting the rapid immaging technique of the Sensovation optical set up. The differenence in ‘τ’ for differt analytes can be used as the discrimination

criterion for the analyte detection.

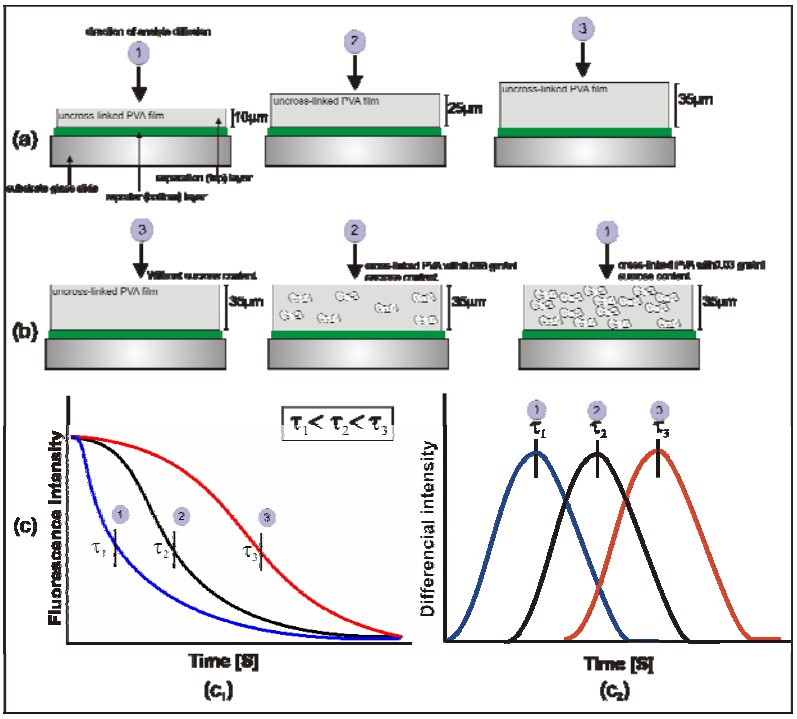


Figure 23: The principle of stacking of polymer layers assigned with basic sensor functions like separation and detection. (a): The thickness of the top polymer layer is varied (10 µm, 25 µm, 35 µm). (b): The sucrose concentration is changed as the polymer modifier agent and top layer thickness is kept constant. (c) The expected fluorescence optical signal for analyte liquid detection according to the top layer modification. The transition time 'τ' is the measured value for analyte differentiation

In (b) part of the figure 25 the time variation is achieved by addition of a polymer modifier and keeping the layer thickenss same. The variation of transports indicated as (1), (2), (3) generates three different 'τ' as shown in the (c_1) part of the figure 25. The exact value of this transition time can be estimated by calculating the differential intensity[7] plots of the data gained in the actual diffusion process shown in figure 25 (c_1). The schematic curves of such plot are depicted in figure 25 (c_2)

[7] *The differential intensity curves taking Figure 27 (C_2) is the data calculated by taking the ratio of the difference between two consecutive data points of the fluorescence intensity curve shown in Figure 5.1 (C_1) of the same data curve. So, essentially the curves shown in Figure 27 (C_2) are the differential ratios of the same curves of the Figure 27 (C_1). This is also the same for all the differential intensity diagrams which follow this discussion.*

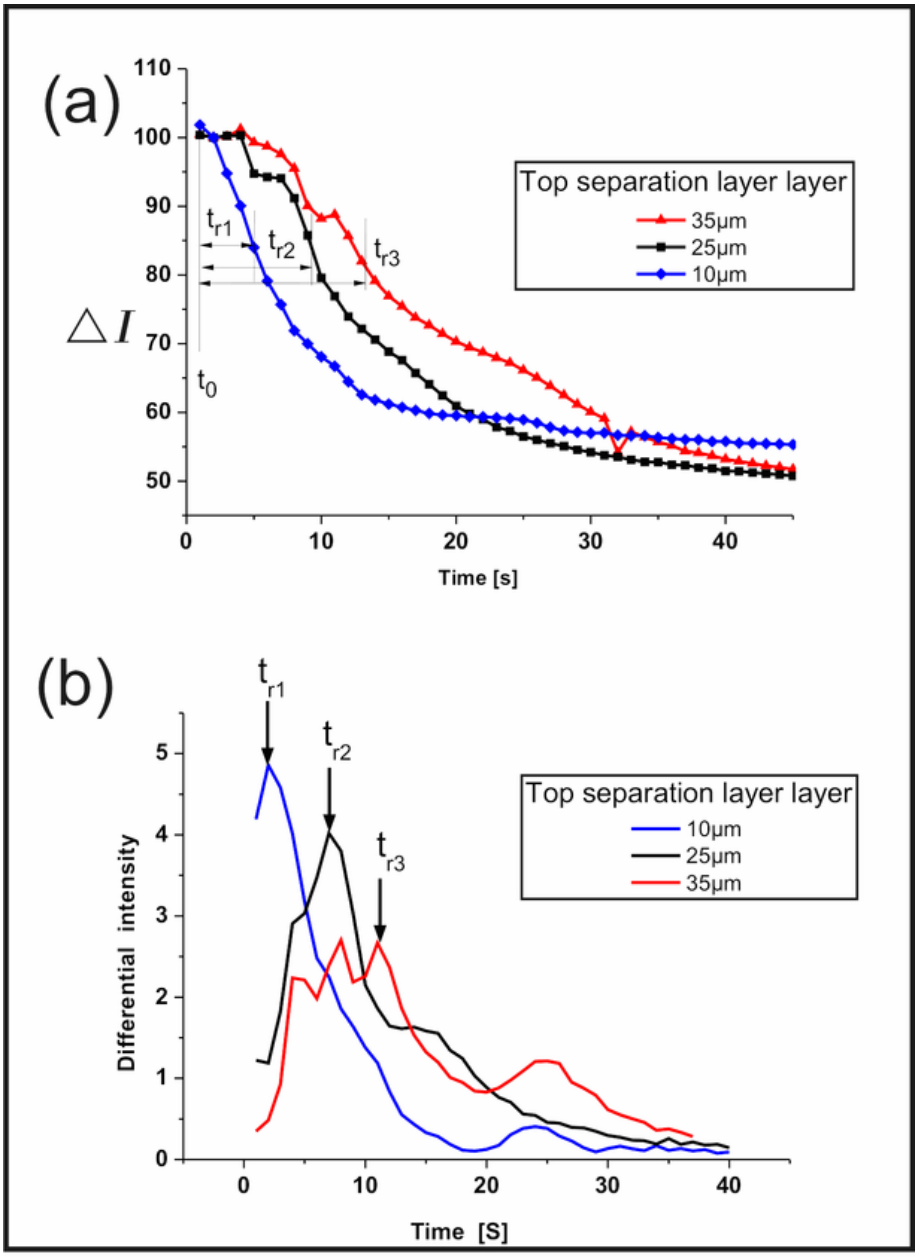


Figure 24: (a) Diffusion signal for 0.25M malonic acid through the top separation layer with varying film thickness. (b) Differential intensity ratio curves of the same data set of figure (a) showing the position of transition time in the diffusion signal

The variation in the top separation layer thickness directly reflected on the change in the transition time of the diffusion process of the analyte species. The 0.25 M malonic acid was taken as the model analyte for these experiments. The PVA top layer was prepared without inclusion of any matrix modifier. Therefore the effect of closely packed polymer matrix chains can be seen directly on the analyte diffusion

times. The lowering of pH should lead to a reduction of the fluorescence intensity of the dye (95). The diffusion is seemed to be complete in about 40s as the fluorescence signal reached to its minimum constant level. The transition times for each film can be estimated as 3 s, 7 s, 10 s for 10 µm, 25 µm, 35 µm thick films respectively.

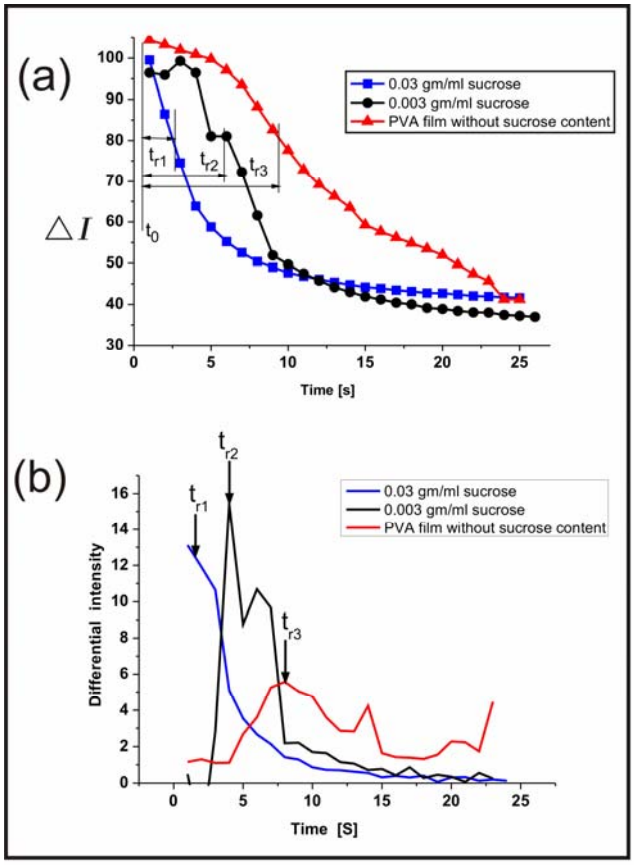


Figure 25: (a): Diffusion signal for 0.25M malonic acid through the top separation layer with varying top layer modifier concentration. (b): Differential intensity ratio curves of the same data set of figure (a) showing the position of transition time in the diffusion signal

The principle is described in figure 25 (b). As the concentration of sucrose in the separation layer was increased the rate of diffusion was also increased. The thickness of the top film was kept constant as ~35 µm. The diffusion transition time for the highest sucrose containing film (0.03 gm/ml) was shortest. It was recorded as 2 s and can hardly be seen in the differential intensity curve. But for the other two films the transition time went on increasing and for the film containing 0.003 gm/ml sucrose it was 4 s and for

pure PVA it was again about 8 s which was in agreement with the data in the figure 24. This shows that the large sucrose molecules may act like a spacer between the closely packed polymer chains. This renders more open space for the transport of the much smaller molecule like malonic acid through the top layer.

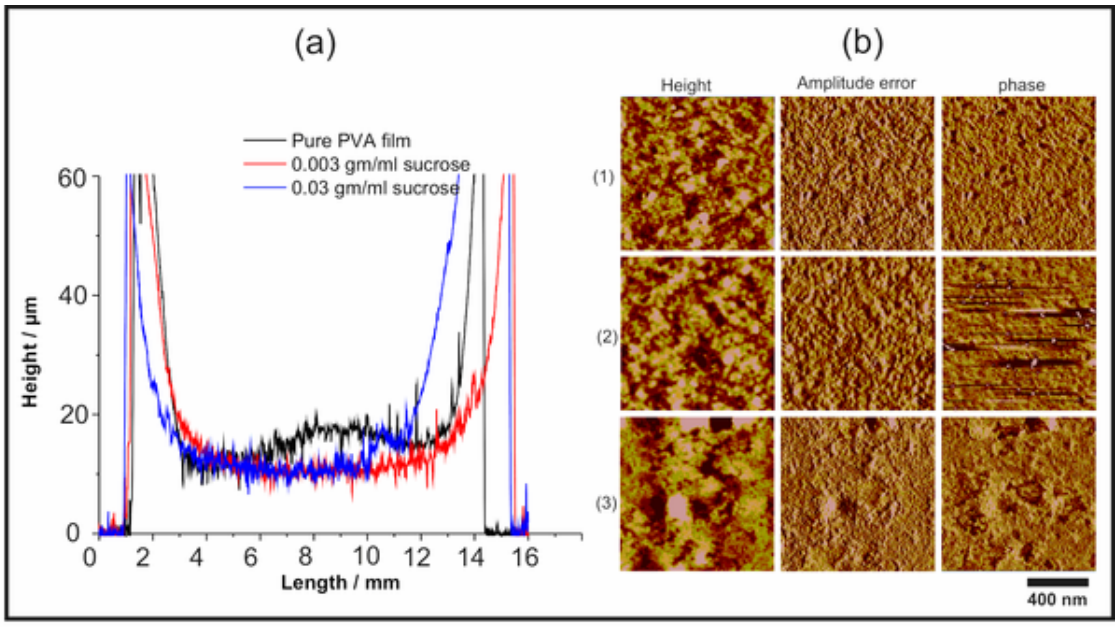


Figure 26: (a): Film thickness measurements of 15 µm thick PVA top layer films with varying concentration of doped sucrose using optical reflectometry. (b): AFM micrographs of three different PVA top layer film surfaces in semi-contact mode. It shows the height, cantilever amplitude damping (amplitude error) and phase difference signals. (1) pure PVA film (2) 0.003 gm/ml sucrose in PVA film (3) 0.03 gm/ml sucrose in PVA film.

In order to investigate the alteration in the diffusion behavior in case sucrose doped DLC, the thickness measurements were carried out using optical reflectometry. Figure 26 a shows the line scans taken through the DLC chip breadth which shows the thickness of the three different DLC with varying sucrose content. These scans show that the sucrose content does not affect the film thickness. Moreover, in figure 26 b AFM micrographs shows the change in the surface texture of the sucrose DLC surface. The smoothening of film surface is observed especially in the amplitude error signal which can be due to more stable PVA films formed with inter cross-linking support of sucrose molecules.

7.2. Development and application of DLCs with local top layer modification

Analytical micro chips must be able to indicate and quantify the presence of an analyte species. For that reason this species must be transported from the analyte liquid to the recognition site in the micro spot. Typically, the spot arrays consist of spots of different indicator and/or recognition sites in order to get different interaction information from different spots. The principle of double layer ChemoChip preparation is depictied in Figure 27.

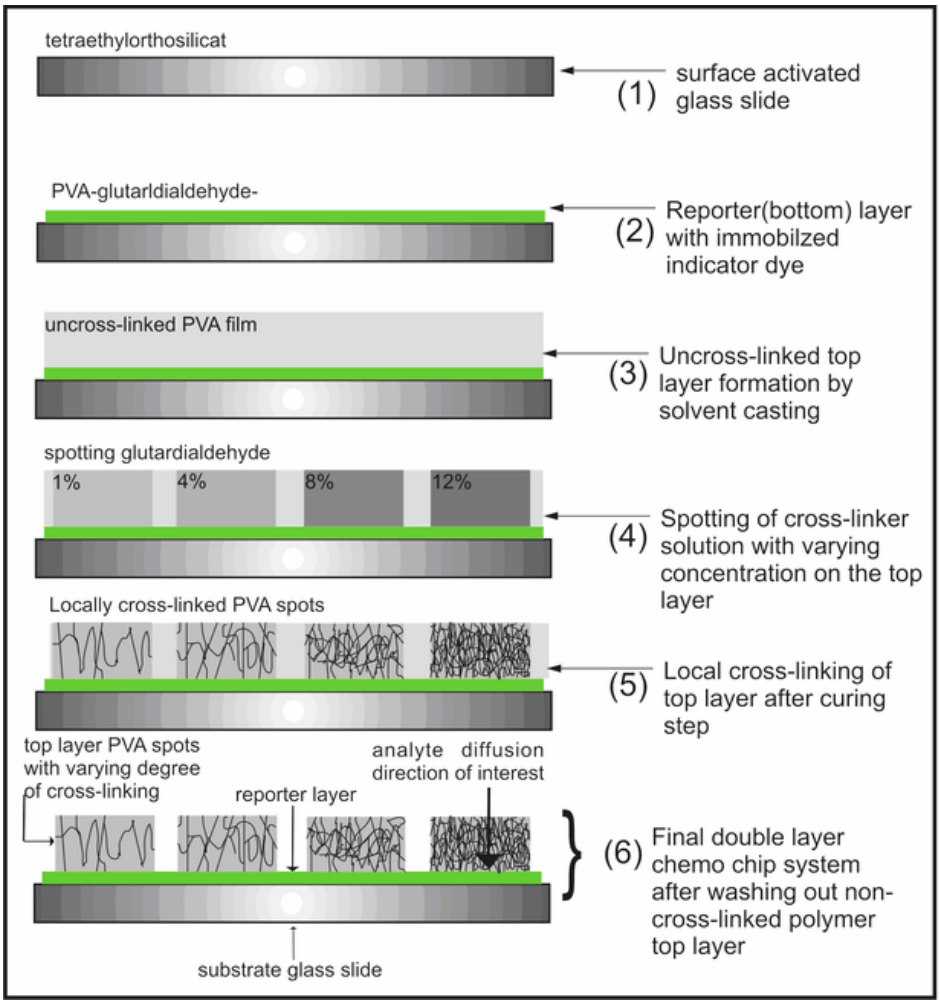


Figure 27: Principle of double layer micro spots for chemo-chip preparation readable by fluorescence imaging

For the preparation of the bottom layer, aqueous solution of fluorescein sodium salt (20 mmol/L) was mixed with aqueous solution of PVA with 5 % (v/v) glutardialdehyde. The films on glass slides (15 mm × 20 mm) were formed by casting techniques (thickness of about 5 – 7 µm) or by spin coating (about 3500 rpm). The glass slides were preactivated by treating them with a 1% v/v tetraethylorthosilicate solution in ethanol for 30 min at 60° C. The immobilization was easily realized by thermal curing. Curing temperature of 120° C for 5 min was sufficient to entrap the dye. Besides dye immobilization, curing leads to a stabilization of the polymer matrix. It was assumed that the thermally induced condensation reactions are responsible for the matrix stabilization. As a result, a water-insoluble polymer fluorescent film was formed, which swells to a gel-state matrix if it was brought into contact with aqueous solutions. The stability of the fluorescence intensity of the dye-doped films was confirmed by rinsing with water and different buffer solutions. After an initial loss of non-immobilized dye molecules, the films have stable fluorescence intensity.

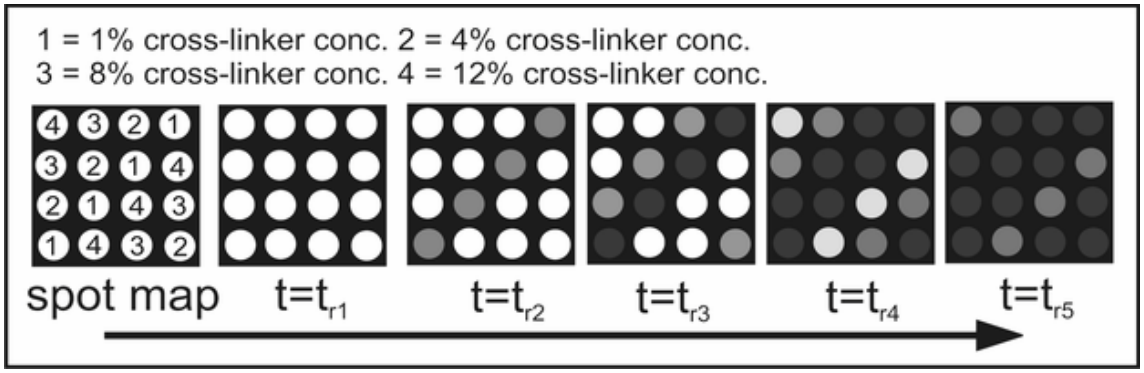


Figure 28: Time-dependent fluorescence imaging in case of permeability-modulated double-layer chemo-chips shown schematically

It was expected that different quantities of film modifiers used would lead to a change of permeability of the top layer in the modified regions. Different time periods those were required for the diffusion of the analyte components into the film stack were expected. Hence the time profile of change of fluorescence signal of the bottom layer depends on the specific properties of the top layer spots. Different spots should respond by different time-dependencies of the fluorescence signal

(schematically shown in Fig. 28).

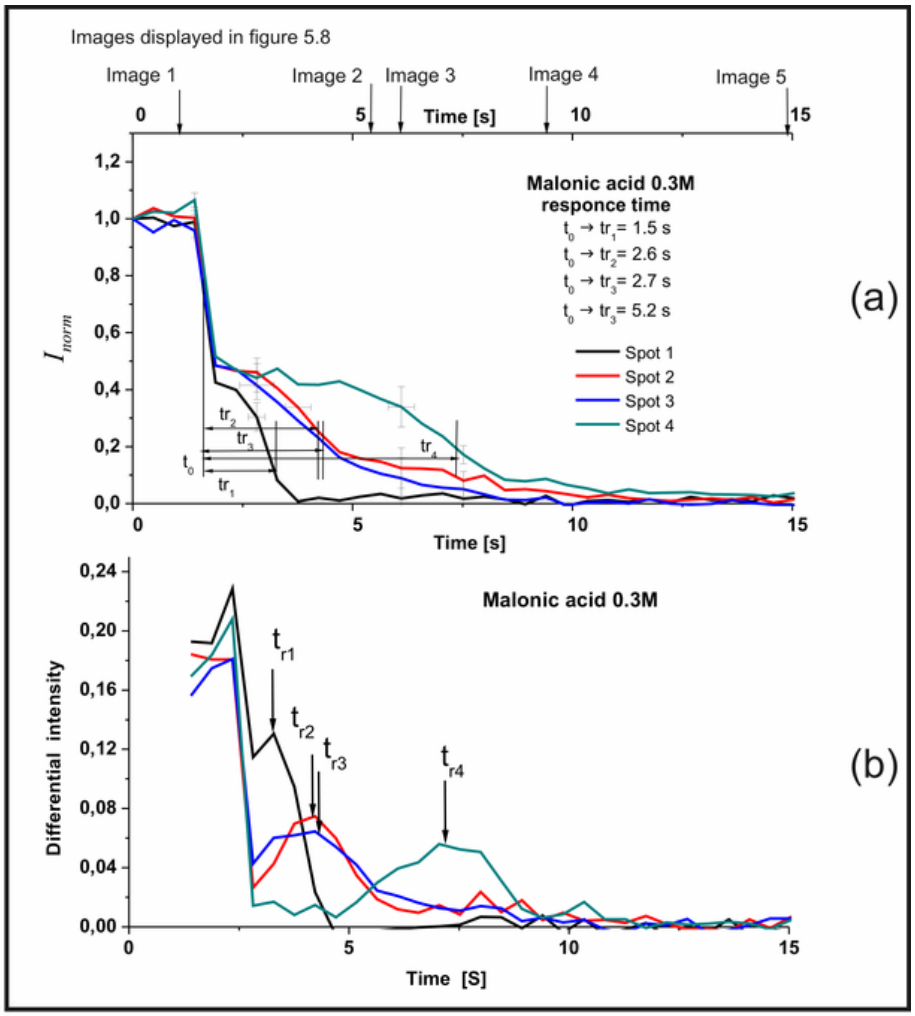


Figure 29: Time-dependent response of 4 micro spots after microfluidic application of an aqueous solution of malonic acid (0.3M); (a) normalized fluorescence signal, (b) Differential intensity curves calculated from the differential ratios of two consecutive data points of the curves in (a). The top layer films used were about 25µm. (200µl PVA solution volume used for the top layer preparation.)

Experiments of the response of chemo-chips with pH-sensitive bottom layer were performed with malonic acid as a substance with moderate acidity (pK_s1 = 2.83). Malonic acid is well soluble in water and in different polar organic solvents and was applied for test purposes. The test solutions contained malonic acid in concentrations of 0.3 mol/L. Double layer chemo-chips consisting of a 5 µm thick PVA bottom layer with a content of 20 mmol/L sodium fluorescein (inside the film) and with a 15-20µm thick top layer carrying 16 spots with four different

concentrations of glutardialdehyde (0.19, 0.38, 0.76, 1.2 mol/L inside the film) were prepared and tested for their response behavior against malonic acid. The general spot pattern for all arrays and the order of spot deposition during the array preparation is shown in figure 29.

First, the DLC was conditioned for two minutes by conducting water through the IMC. In this time period, the film swelling was completed and the initial conditions for the analyte applications were well defined. The sample volume of 200 µL of malonic acid solution was applied with a flow rate of 40 µL/s into the main liquid stream. The time-dependent fluorescence response of the DLC was registered by sequential fluorescence imaging with an image aquisition rate of 2 frames per second.

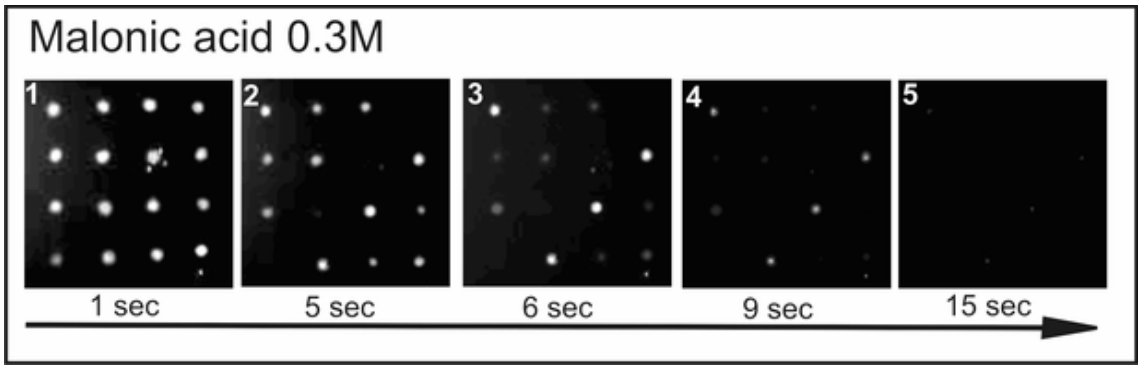


Figure 30: Time series of array response after microfluidic application of an aqueous solution of malonic acid (0.3 M), fluorescence read-out at 530 nm

All spots showed a delayed change in the fluorescence intensity compared to the unaltered reference intensity (Figure 29). In addition all spots showed a significantly fast decrease in fluorescence after about five seconds. This first drop in fluorescence intensity (about 50%) is nearly independent of spot composition and is obviously caused by the substitution of the carrier liquid by sample liquid inside the measurement cell or the fast pH change. The first (constant) fluorescence drop is followed by a second process with a typical transient behavior: In the beginning, the intensity change is slow, but becomes faster as the difussion progrsesses. During the further process, the fluorescence intensity is drastically reduced, down to about 15% or less. The process is marked by a specific duration that means a significant

transient time. This transient time is different for different spots (1.5 s for 1% , about 2.6 s for 4% 2.7 s for 8%, and 5.2 s for 12% of glutardialdehyde). That hints to the realization of different permeabilities in the top layers of the spots by the presence of different concentrations of cross linking agents during the preparation. The differences in the response behavior are well reflected by the evolution of fluorescence images of the whole array (Fig. 30).

The differences in the rate of fluorescence change stands for a significant modulation of the molecular transport in the top layer by the cross linking agent. It demonstrates that the chemochip spots with different transport behavior can be realized by the micro spotting techniques for DLCs.

The action of phenanthroline hydrochloride on DLCs as a second model substance was investigated in order to have an alternative chemical species to check the response found with malonic acid. The type of applied DLCs were the same as in the case of malonic acid investigations and the procedure of application of the analyte solutions was also followed as described previously. The qualitative character of the obtained fluorescence responses was the same as in the experiments with malonic acids. (Fig. 31).

The equal initial fluorescence drop was found to be much smaller (less than 15%) than in the case of malonic acid. For spot 1 (1% cross linker), it is impossible to distinguish between the first and second transition. That means the second transition is also fast in this case and amounts to about two seconds only. In contrast to malonic acid, a much broader distribution of the second transition time is observed. It increases over 10.5 s (2nd spot, 4% glutardialdehyde) and 25.2 s (third spot, 8% glutardialdehyde) up to 31.2 s of the fourth spot (12% glutardialdehyde). This observation must be interpreted as a stronger effect of the cross linker on the permeation of phenanthroline hydrochloride through the top layer. The differences in the effect of cross linker on the transient times can obviously not only be explained by the permeation of the hydronium and hydroxide ions but by the transport conditions for the larger ions. The differences in the transition times of phenanthroline hydrochloride are also illustrated by the fluorescence images of the whole array for different times after sample application (Fig. 32). Time series of array response after microfluidic application of an aqueous solution of

phenanthroline hydrochloride (0.3 M), fluorescence read-out at 530 nm, time interval of 0.5 s.

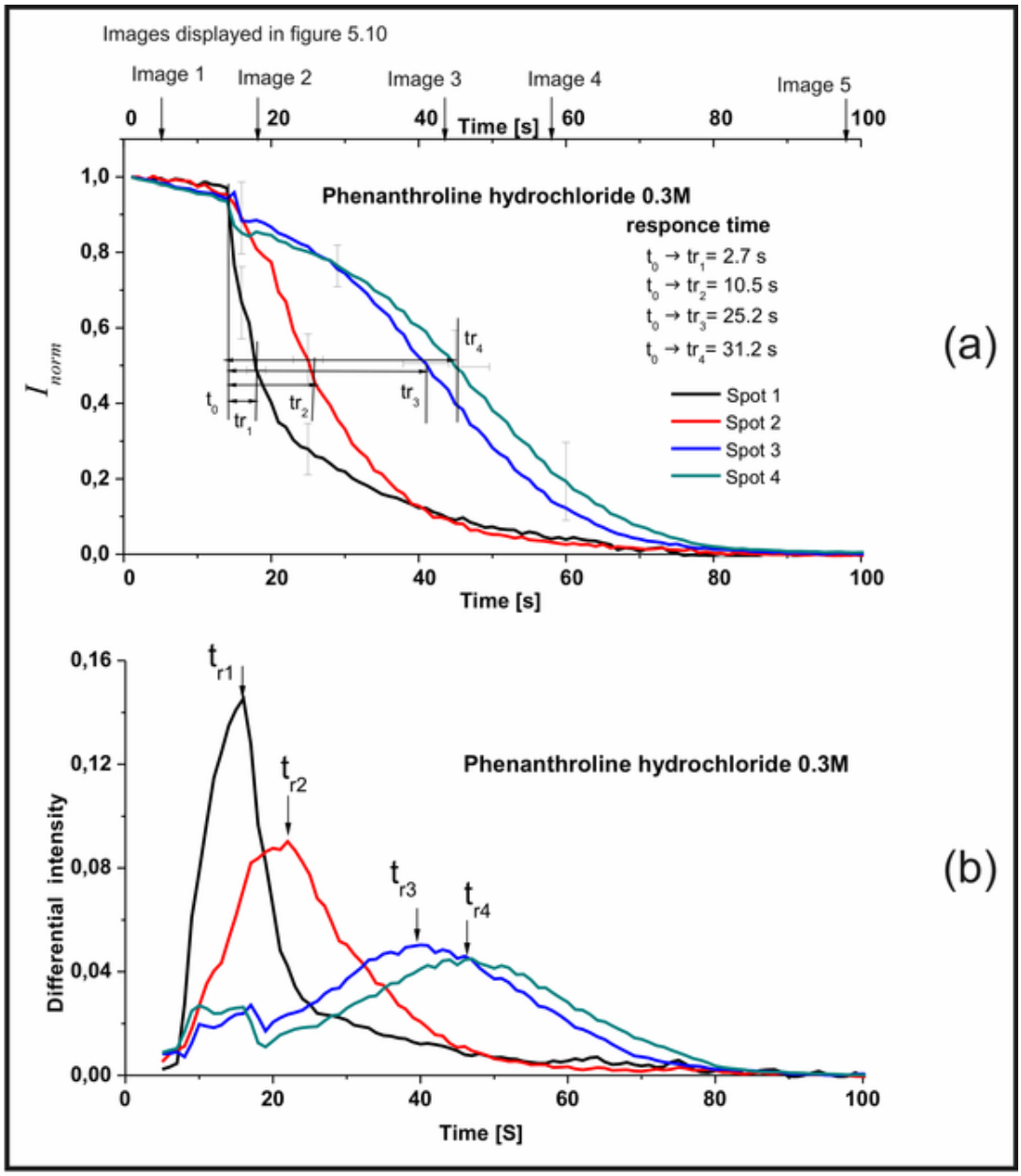


Figure 31: Time-dependent response of 4 micro spots after micro fluidic application of an aqueous solution of phenanthroline hydrochloride (0.3M); (a) normalized fluorescence signal, (b) Differential intensity curves calculated from the differential ratios of two consecutive data points of the curves in (A). The top layer films used were about 25 µm. (200 µl PVA solution volume used for the top layer preparation.)

The experiments with phenanthroline hydrochloride confirm the increase in transition time with increasing concentration of cross linking agent in the top layers of the micro spots. The transition times of phenanthroline hydrochloride are considerably higher than in the experiments with malonic acid solutions. This observation demonstrates specific differences in the transport rate of both substances through the top layers of the double layer system.

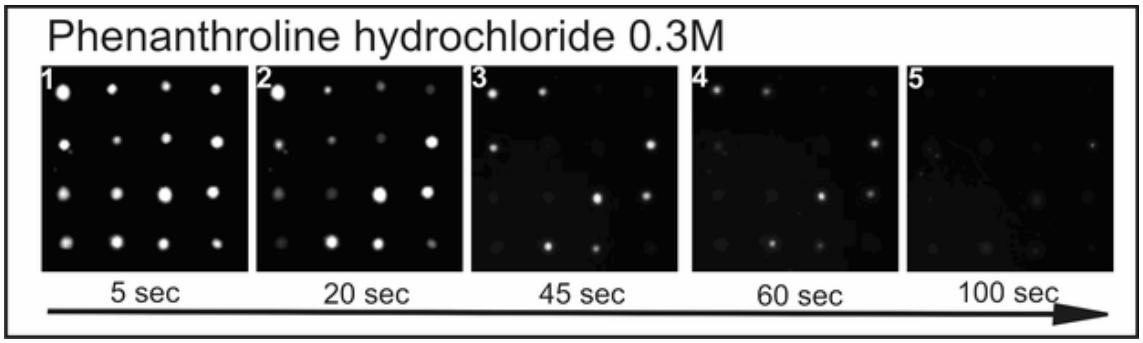


Figure 32: Time series of array response after microfluidic application of an aqueous solution of phenanthroline hydrochloride (0.3 M), fluorescence read-out at 530 nm

7.3. Development and application of DLCs arrays with higher spot height

The array prepared by spotting matrix modifier was characterized with the help of optical profilometer (sec3.2.4). It was remarkable that although the PVA cross linker glutardialdehyde solution used for spotting was increased in the order 1%- 4%- 8%- 12% (v/v).

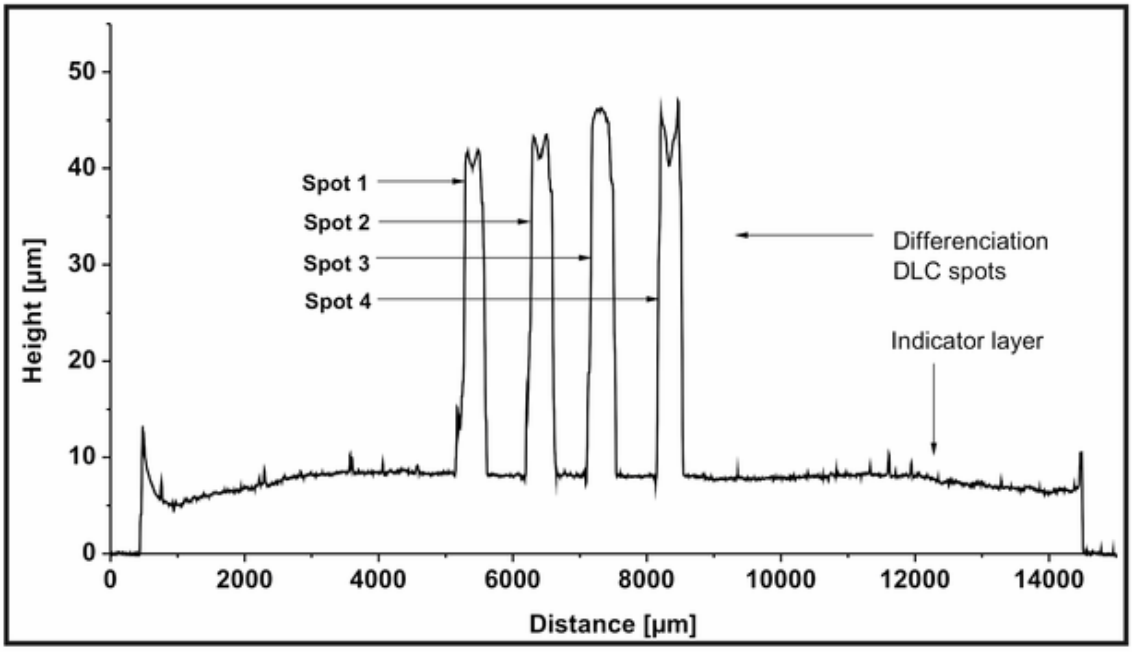


Figure 33: The DLC spot profile taken with the optical reflection profilometer

This has not affected the spot height in the final form of DLC array. This confirms that the diffusion signal observed for each spot is coming out of the change in degree of cross-linking inside the spot. And not because of the spot height difference due to varying amount of PVA material hold together in the matrix with different cross-linker concentration.

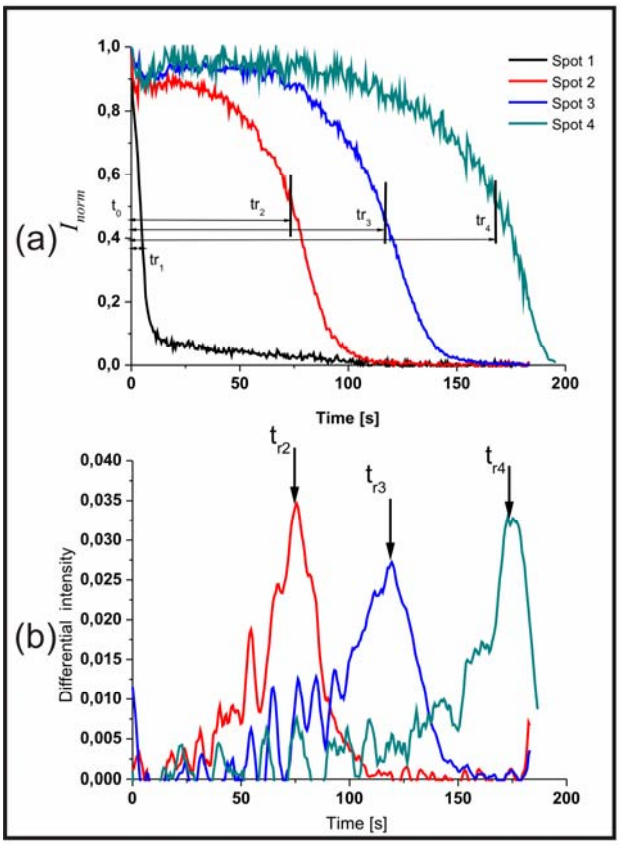


Figure 34: Time-dependent response of 4 micro spots after microfluidic application of an aqueous solution of phenanthroline hydrochloride (0.3 M); (a) normalized fluorescence signal, (b) Differential intensity curves calculated from the differential ratios of two consecutive data points of the curves in (a). The Top layer films used were about 40µm. (300µl PVA solution volume used for the top layer preparation.) The spots 1, 2, 3, 4 were prepared by 1%, 4% 8%, 12% (v/v) of glutardialdehyde respectively.

The Transition time response for the 0.3 M phenanthroline hydrochloride became more distinctive by the application of thicker differentiation top layer. The thickness increase of the top layer was realized by simply using more amount of polymer solution in the solvent casting process. The actually spot height was estimated using the spot optical profile described before (Fig. 34. The t_{r1} for spot one was passed very quickly and therefore could not be recognized. But the other three spots prepared with the 4%, 8% and 12% glutardialdehyde showed a good distinction for t_{r2}, t_{r3} and t_{r4}. These transition times can be well estimated as 75 s, 125 s and 175 s. The exact value of

the transition time was estimated from the figure 34 b). The three maxima for the transition point are seen very well separated. This optimization in the separation layer thickness variation can be helpful for the application of the DLCs as the on chip chromatographic device.

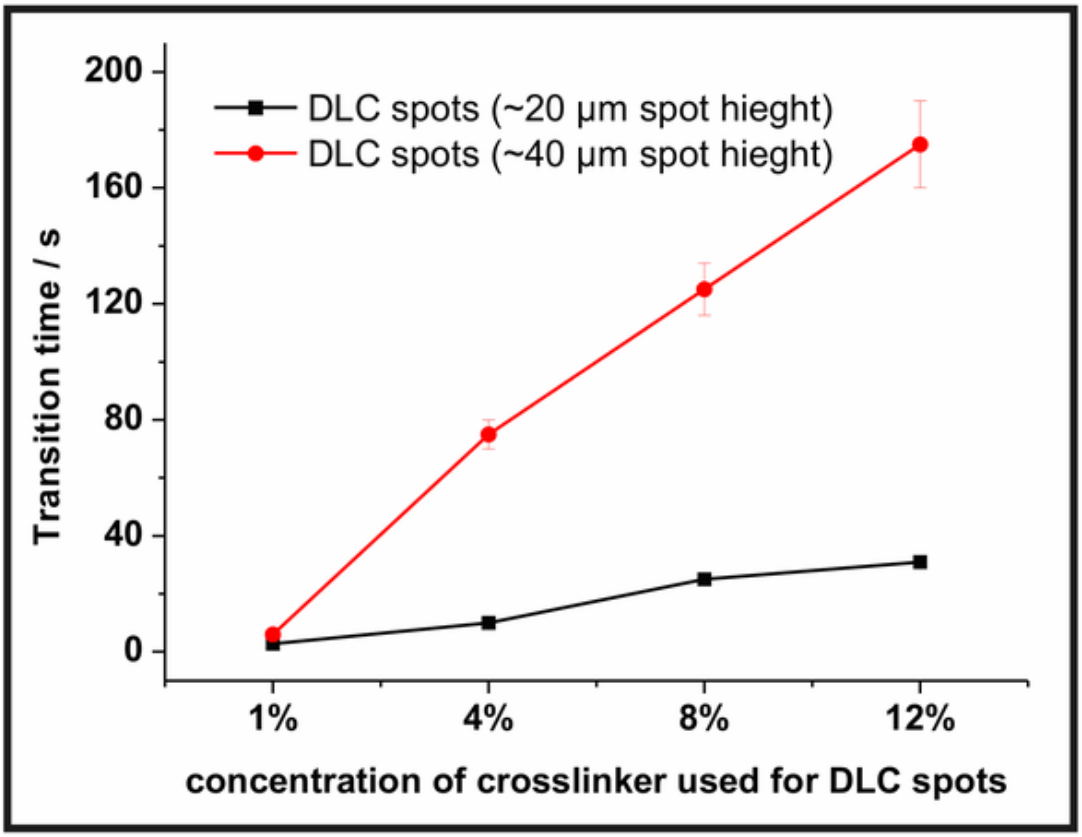


Figure 35: Comparison of the change in the transition time of the phenanthroline (HCl) through the DLC spots prepared with for varying cross-linker concentration and amount of PVA used for its preparation (spot height).

The change in the transition times as a function of the DLC spot degree of cross-linking has been shown in figure 35 The increase in the height of the DLC spots (as compared to that of in section 5.2 (~20 µm→~40 µm), enhanced the transition times difference (distribution) among the four types of DLC spots. The thinner DLC spots (Figure 35 black legend) has the difference of about 15s and with the thicker DLC spots it increase to about 170 s between the least cross-linked spots and the highest cross-linked spots. This enhances not only the separation power but also give additional

recognition ability to the DLCs by rendering a larger window of real time analyte separation.

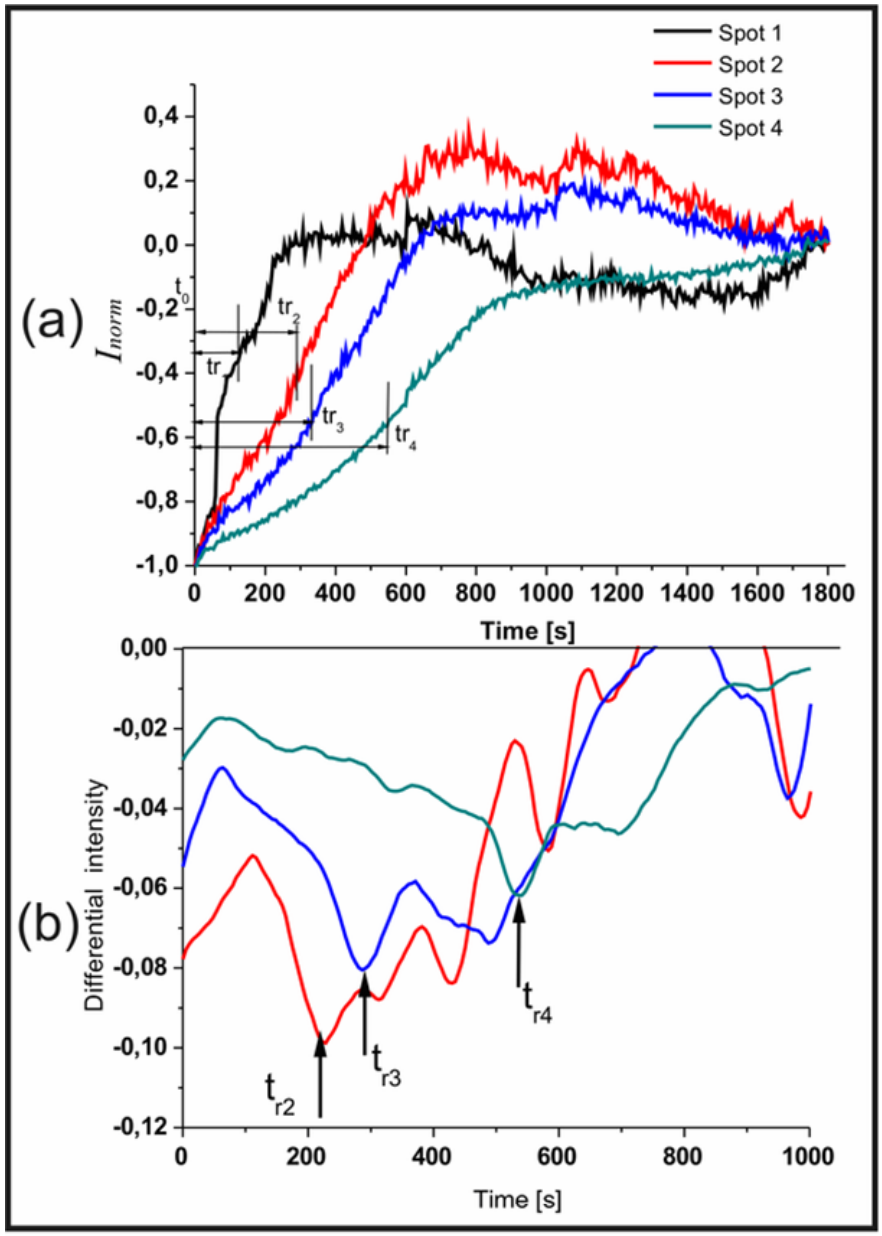


Figure 36: Time-dependent response of 4 micro spots after microfluidic application of an aqueous solution of triethylamine (0.3 M) in 1:1 ethanol, water mixture as solvent (a): normalized fluorescence signal, (b): Differential intensity curves calculated from the differential ratios of two consecutive data points of the curves in (a). The Top layer films used were about 40 µm. (300 µl PVA solution volume used for the top layer preparation) The spots 1, 2, 3, 4 were prepared by 1%, 4% 8%, 12% (v/v) of glutardialdehyde respectively.

The triethylamine (TEA) in 1:1 ethanol water solution was applied on the DLC (Fig. 37). Its being basic in nature caused increase in the fluorescence intensity of the sodium fluorescein dye. Therefore the diffusion curves obtained to be inverted in direction as compared with the acidic solution of phenanthroline hydrochloride. In the case of TEA the diffusion process was prolonged and took about 25 min to be completed. This may have caused due to less swelling of PVA spots as the solvent medium has lower polarity than the pure water. But the spot response once again was directly related to the degree of cross-linking of the top separation layer spots. The four transition times for the TEA curve can be estimated as 100 s, 220 s, 250 s and 575 s for the four spots respectively. The differential intensity curves also were inverted due to opposite signal nature of the DLCs towards basic solution as the calculation protocol was kept same, which was used for the earlier acidic solution experiments.

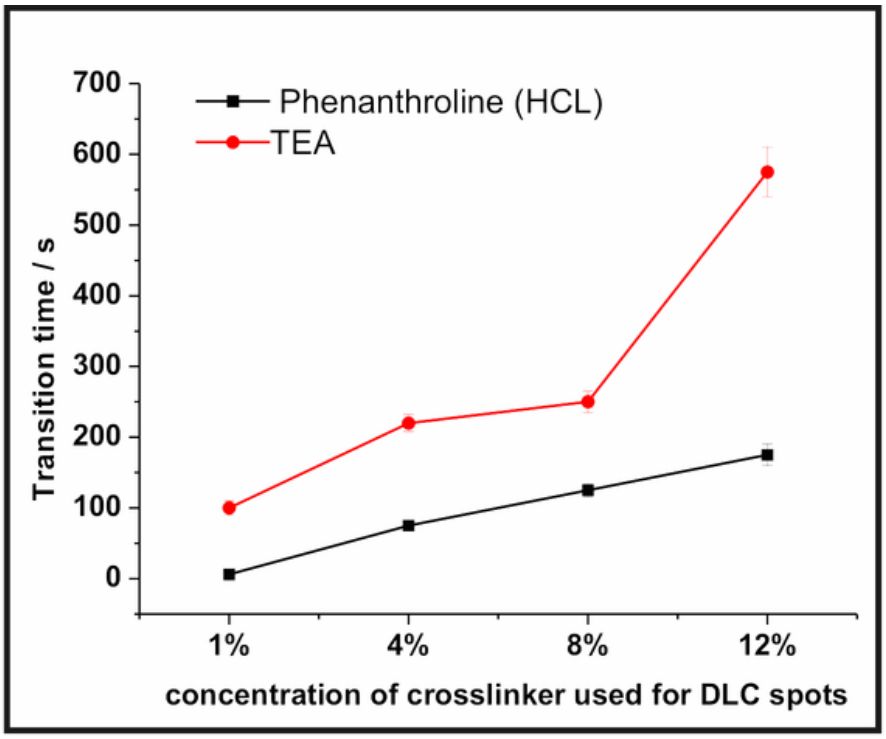


Figure 37: The change in the transition time for the DLC spots with varying cross-linker concentration for phenanthroline (HCl) dissolved in pure water and TEA dissolved in 1:1 water-ethanol mixture.

The transition time comparison between the two model analyte has been shown in the figure 38. The clear distinction in the transition times of the two analytes can made here. It can be seen that the discrimination ability of the DLC is increased with the increase in the degree of cross-linking. The spot prepared with the 12% v/v of the cross-linker solution shows the highest transition time difference of about 420 s of the two analytes.

The application of the mixture of the two acidic and basic analytes liquids received a dual response from the DLC. The figure 39 shows the direct diffusion signal of the mixed species[8].

The analyte species in this case is a reaction solution involving an acid-base reaction with number of reaction products. The diffusion obtained in this case does not indicate the separation of multiple numbers of product species but only the basic followed by acidic diffusing species qualitatively.

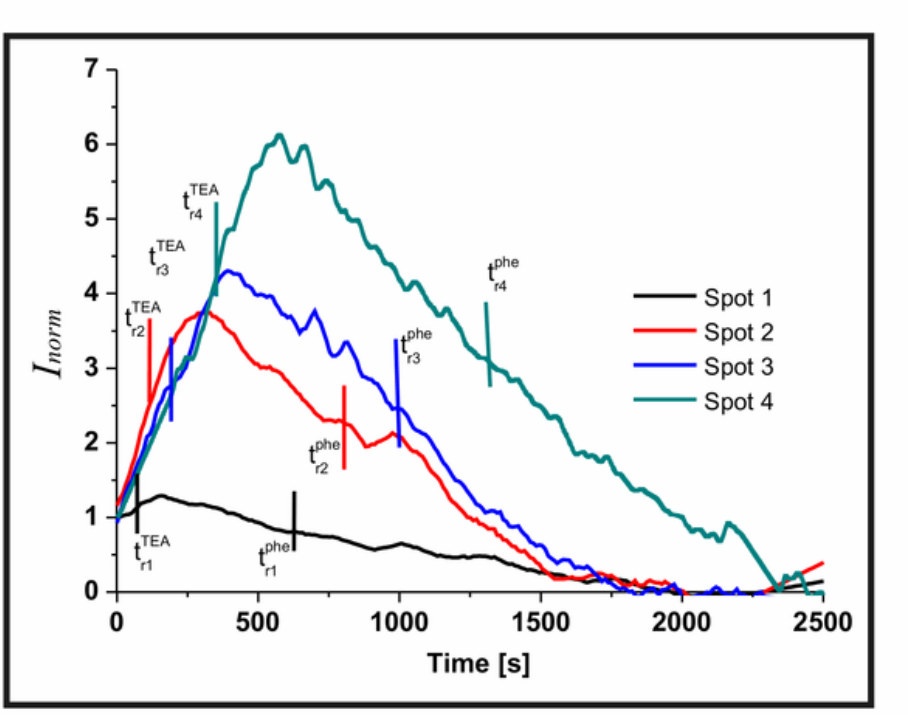


Figure 38: The equivolume phenanthroline hydrochloride and triethylamine solutions (0.3 M each) mixture was prepared in 1:1 ethanol water as solvent. This solution was

[8] *The curves should not be confused with the differential intensity curves due its similar appearance of the plots.*

applied on the DLC and the diffusion process was recorded. The figure shows the diffusion curves for the mixed acid-base solution.

The pH meter reading of the reaction solution showed the pH of 2.5. Therefore the solution has a strong acidic character, yet in the diffusion curves there is the signal (fluorescence increase) pertaining to the basic species first, which increase the fluorescence intensity when compared with the previous reading (Fig. 34 and Fig. 36. This indicates the ability of the DLC to separate the mixed species and detect them qualitatively. However, at this stage it is not possible to predict about the exact mechanism obeyed by this separation process. The maximum in the diffusion curves arises due to the delayed diffusion of acidic species. It is fastest for lowest cross-linked spot and increases with increase in the cross-linking degree. The shift in the position of the maxima for the four types of DLC spots has been shown in figure 39

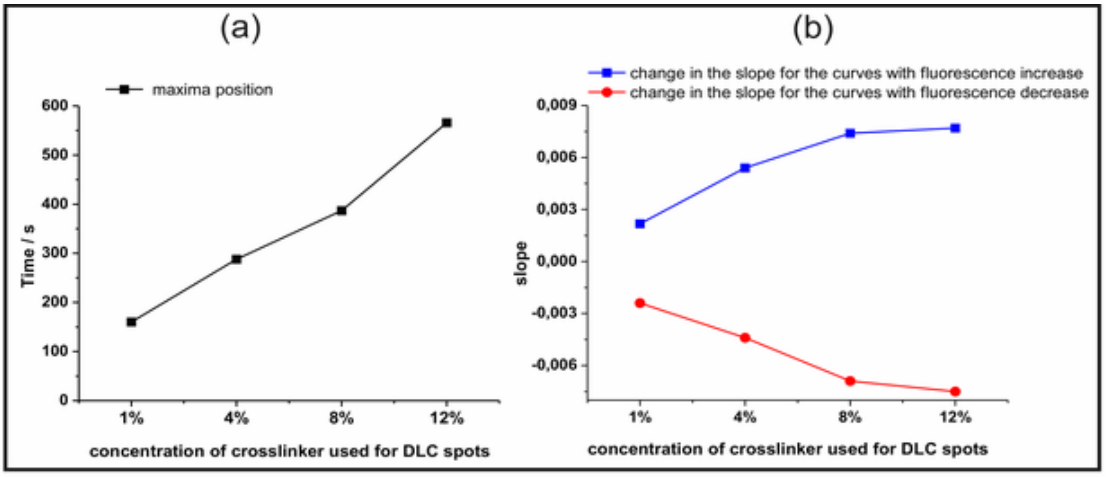


Figure 39: (a) Shift in the position of the fluorescence curve maxima in figure 38 as a function of the cross-linking degree of the DLC spots. (b) Change in the slope of the fluorescence curves for the mixed species shown in figure 39.

It also demonstrates the trend in the shift of transition maxima. A good range of distribution of transition times of about 425 s over all the DLC spots can be seen. The comparison of the slopes of the two sides of the curve can be given to discriminate between the two types of signals in more detail. In figure 39 b the change of the slope of the two types of the fluorescence curves has been shown. The change of slope of in this case stands for the difference in the diffusion rate as function of cross-linking degree of

the spots. Here it can be observed that the DLC spot response for the mixed analytes is different than it is for its individual investigation. However the spots still are able to discriminate the species, which can be demonstrated by the change in the slopes of the respective curves reflected figure 39 b.

As an another example of mixed analyte application, 0.3 M solution of malonic acid and phenanthroline hydrochloride were mixed in 1:5 proportion and used as the anlyte liquid in the DLC difussion experiment. In this case, two different transiotion points were seen in a single difussion curve. These transition points can be seen more clearly in the differential intensity plots. The single curves of spot 3 and spot 4 from figure 40 (a) shows two maxima in the figure 40 (b) which represents the existance of two distinct transition points in a single difussion process. With the help of these mixima in spot 3 and spot 4 curves (differential intensity) the two separate transition points for the two mixed species can be determined quantitatively. For spot 3 the first transition point comes at 12.5 s which can be assigned to the malonic acid difussion process and the next transition point in the same curve appears at the 68.7 s.

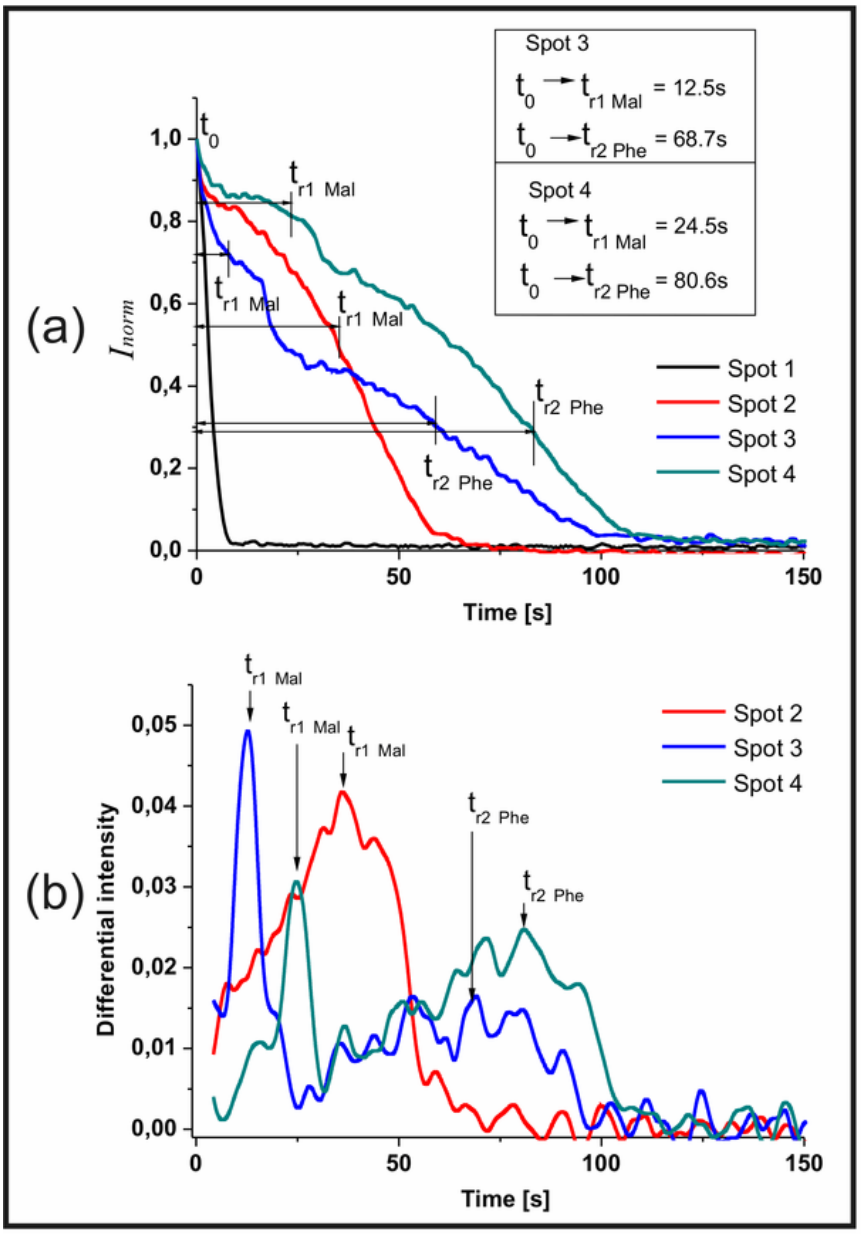


Figure 40: Difussion response of 0.3 M malonic acid and phenanthroline hydrochloride aqueous solutions mixed in proportion 1:5 respectively. (a) normalized fluorescence signal, of actual analyte difussion signal recorded using Sensovation set up. (b) Differential intensity curves calculated from the differential ratios of two consecutive data points of the curves from figure 'a'. The curves shows two clear separated transition signals for the mixed species as analyte liquids for spot 3 and 4.

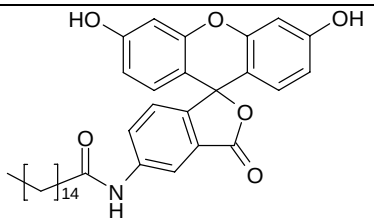
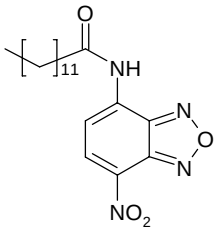
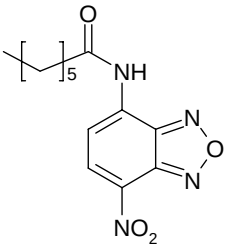
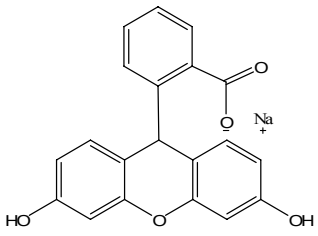
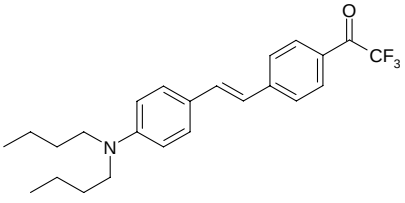
In case of spot 4 the transition times have increased to 24.5 s for malonic acid and 80.6s for phenanthroline hydochloride. This can be due to increase in the

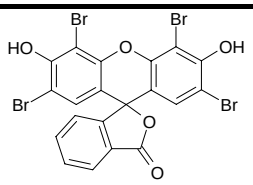
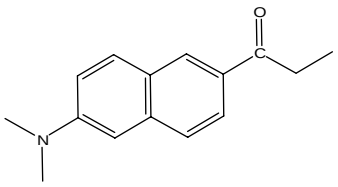
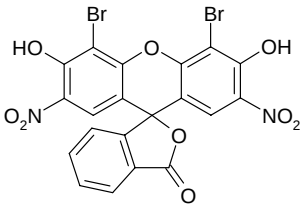
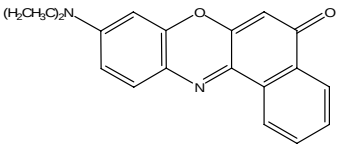
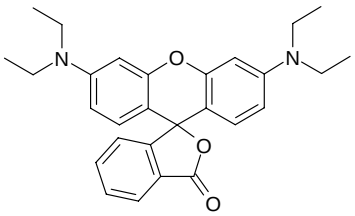
crosslingking degree form spot 3 to spot 4. However in the case of spot 1 and spot 2 such double trasition curves are not seen. Therefore the dgree of cross linking may play the critical role in such separation process, which is appropriate in the case of spot 3 and 4 which are able to distinguish between the two mixed species transitions.

8. Conclusions

The project “ChemoChips” proved the principle of array based analysis of liquid probes for applications in an non biological environment. Therefore liquid analytes were investigated with fluorescent labeled single layer as well as double layer ChemoChips. A special setup was developed in cooperation with the collaborating partners to proceed the optical experiments. Single layer chips as well as double layer chips were prepared by application of solvent casting, doctor blading and micro pipetting techniques. Beside the fluorimentric characterization various techniques were used to characterize the received films and spots arrays. Exemplary solvents and substances were used for the characterization of the different array response. Individual spot intensities as well as array patterns were analyst with respect to their information content. The application of non specific analyte recognition inside the dye doped matrix in combination of an multiple responding array was proved and a complex interaction network was proposed as mechanistic principle therefore. Commercially available beverages were investigated whereas the compositions were very similar. As a result the developed array respond individually to the different beverages. The developed double layer principle opens up a wide range of new application. The combination of an adjustable selection layer on top of a reporting array allows to formation of a new type of array from here on.

Appendix I: Dyes

Dye Number	*Name (commercial name and source)*	*Structure*
1	H110, Molecular Probes Inc. Leiden, The Netherlands, CAS- 73024-80-3 (Abs: 497 , Em: 519)	
2	N678,Molecular Probes Inc. Leiden, The Netherlands, CAS- 96801-39-7 (Abs: 467 , Em: 536)	
3	N316, Molecular Probes Inc. Leiden, The Netherlands, CAS- 88235-25-0 (Abs: 467 , Em: 539)	
4	Fluoresceine (sodium salt) Fluka Chemie GmbH. Switzerland. CAS- 518-47-8 (Abs: 490 , Em: 520)	
5	ETH 4003, Fluka Chemie GmbH. Switzerland. CAS- 192190-91-3 (Abs: 550, Em: 630)	

6	Eosin Y, Merck KgaA. Darmstadt, germany. CAS- 17372-87-1 (Abs: 552 , Em: 610)	
6[9]	Prodan Merck KgaA. Darmstadt, germany CAS-70504-01-4 (Abs: 361,Em: 498)	
7	Eosin B, Neolabs GmbH. Heidelberg, Germany CAS- 548-24-3 (Abs: 361, Em: 498)	
7[10]	Nile red Neolabs GmbH. Heidelberg, Germany CAS- 7385-67-3 (Abs: 552, Em: 663)	
8	Rhodamine B Merck KgaA. Darmstadt, germany. CAS- 81-88-9 (Abs: , Em:)	

[9] *Dyes tried out for immobilization shown in figure 3.4a*
[10] *Dyes tried out for immobilization shown in figure 3.4a*

Appendix II: Spot signal reproducibility data for the model experiment of phenanthroline-hydrochloride

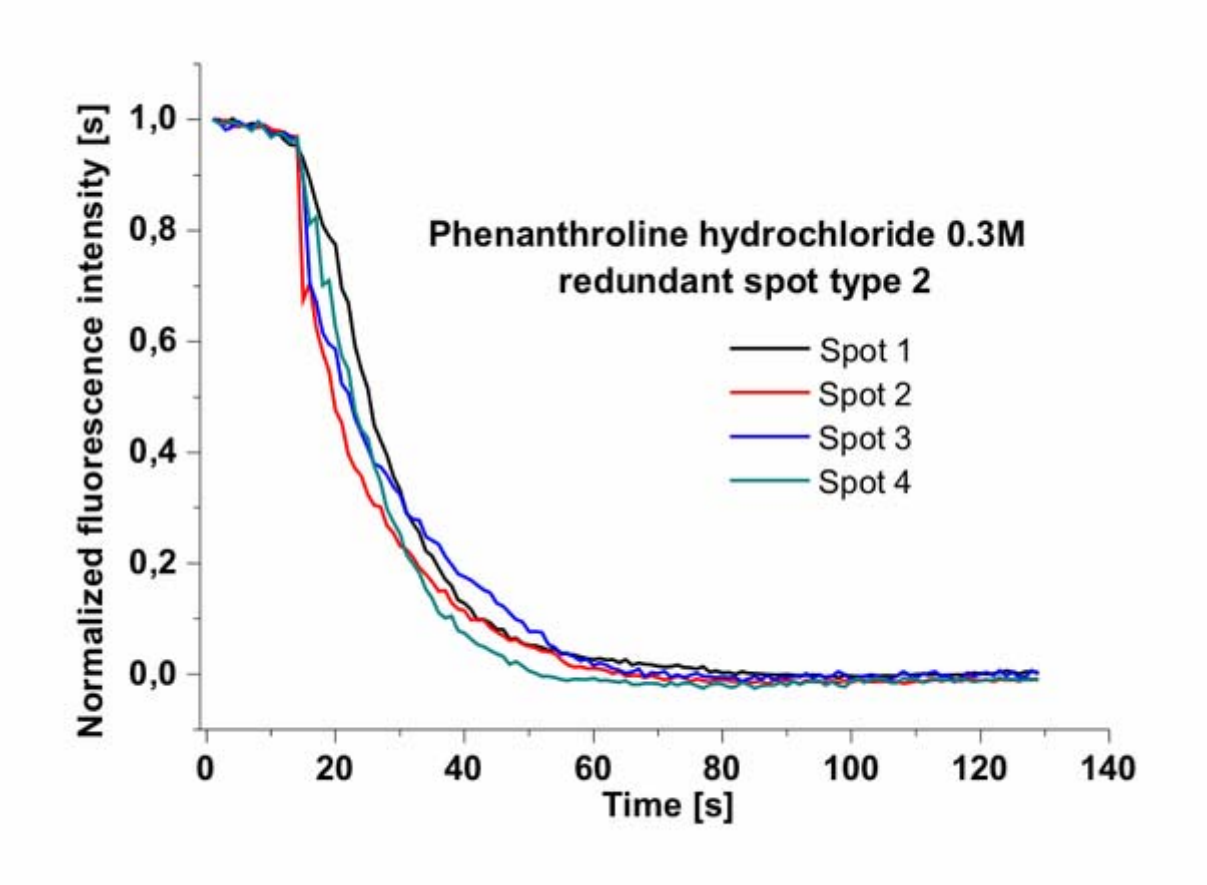


Figure 1: Spot type 2 signal reproducibility for all the four redundant spots in the array.

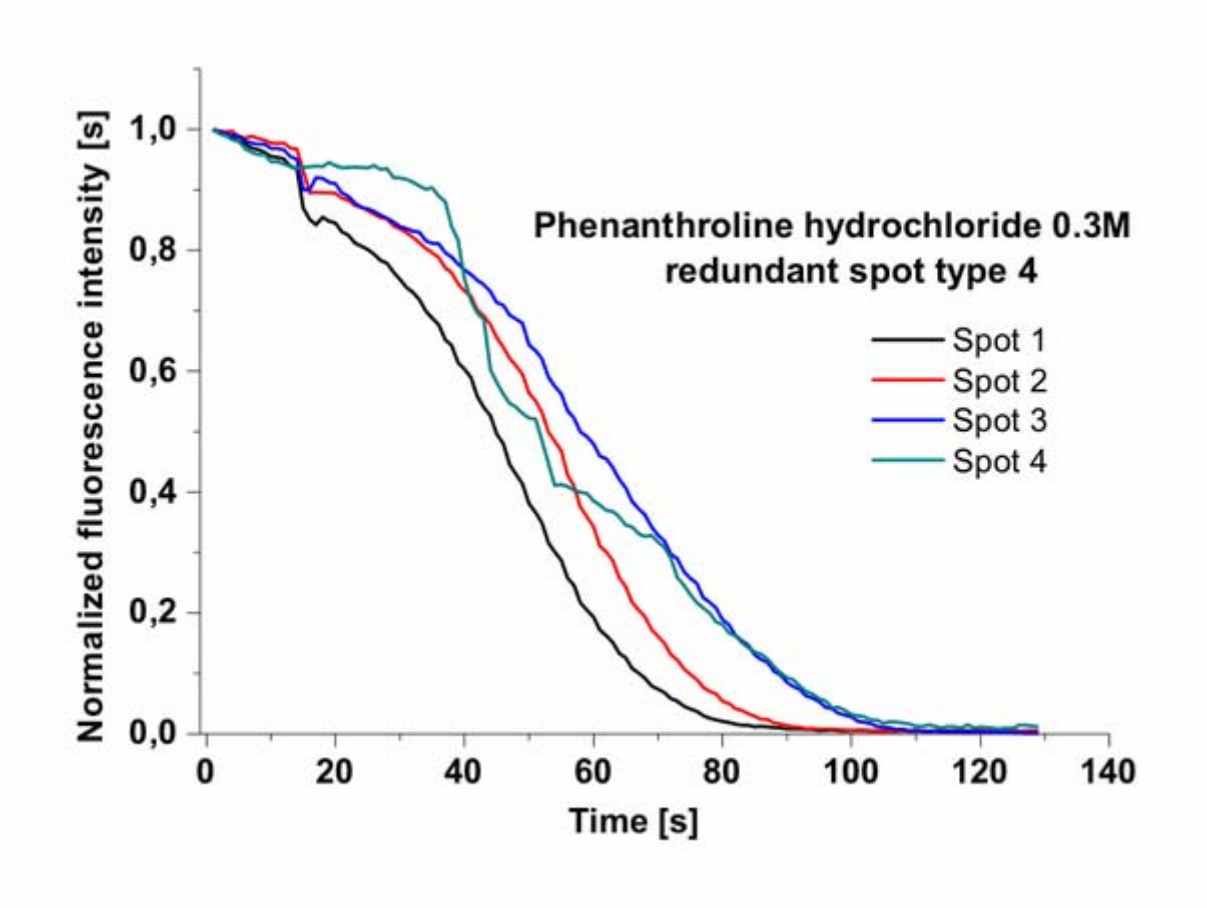


Figure 2: Spot type 4 signal reproducibility for all the four redundant spots in the array.

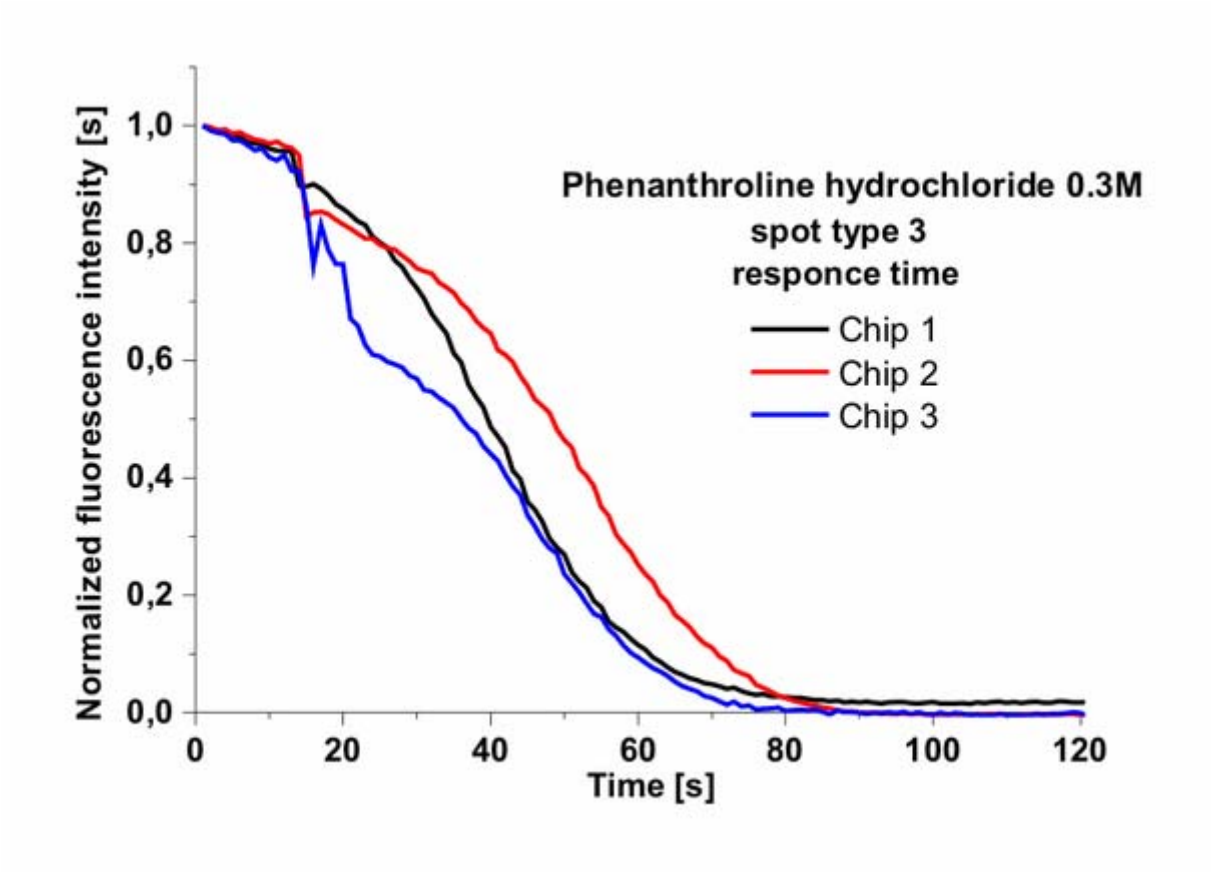


Figure 3: Spot type 3 signal reproducibility for three different chips under same experimental conditions.