Version 26/05/09

Centre Interuniversitaire de Microélectronique de Grenoble

"BioChips ": micro-fabrication and characterization of DNA based-microarrays on glass

This practical work is organized in two parts that will take place at the Interuniversity Center for MicroElectronics (CIME), Minatec BCAi, 3 parvis Louis Néel, Grenoble (Tram B: Cité internationale)

Day 1 (4 hrs) – Covalent grafting of DNA probes on silanized glass slides

Day 2 (4hrs) – Hybridization with fluorescently labeled target DNA.
Functional characterization by fluorescence scanning and quantitative image analysis.

This lab work was prepared by Michel Labeau and Jean-Edouard Mendez at Grenoble INP Phelma with the help of Pierre Barritault, Antoine Huang, David Peyrade and Philippe Pelletier at the Laboratoire d'Electronique et des Technologies de l'Information at the CEA-Grenoble. It was updated and put together by Franz Bruckert and Marianne Weidenhaupt (Grenoble INP Phelma).

MATERIALS USED

The DNA chip is produced on commercial glass slides from Genewave (http://www.genewave.com/) specially manufactured for fluorescence based microarray applications.

Amplislide ™ A (Genewave)

All specifications of the glass slides used are given on the product sheet (p.3).

The glass surface is already functionalized with an aminosilane layer that will be used to covalently attach NH_2-DNA probes via a glutalaldehyde linker (see chemistry in Appendix 1).

Amplislides exhibit a unique fluorescence-amplifying, thin film optical coating that allows a 10 to 20 fold signal enhancement.

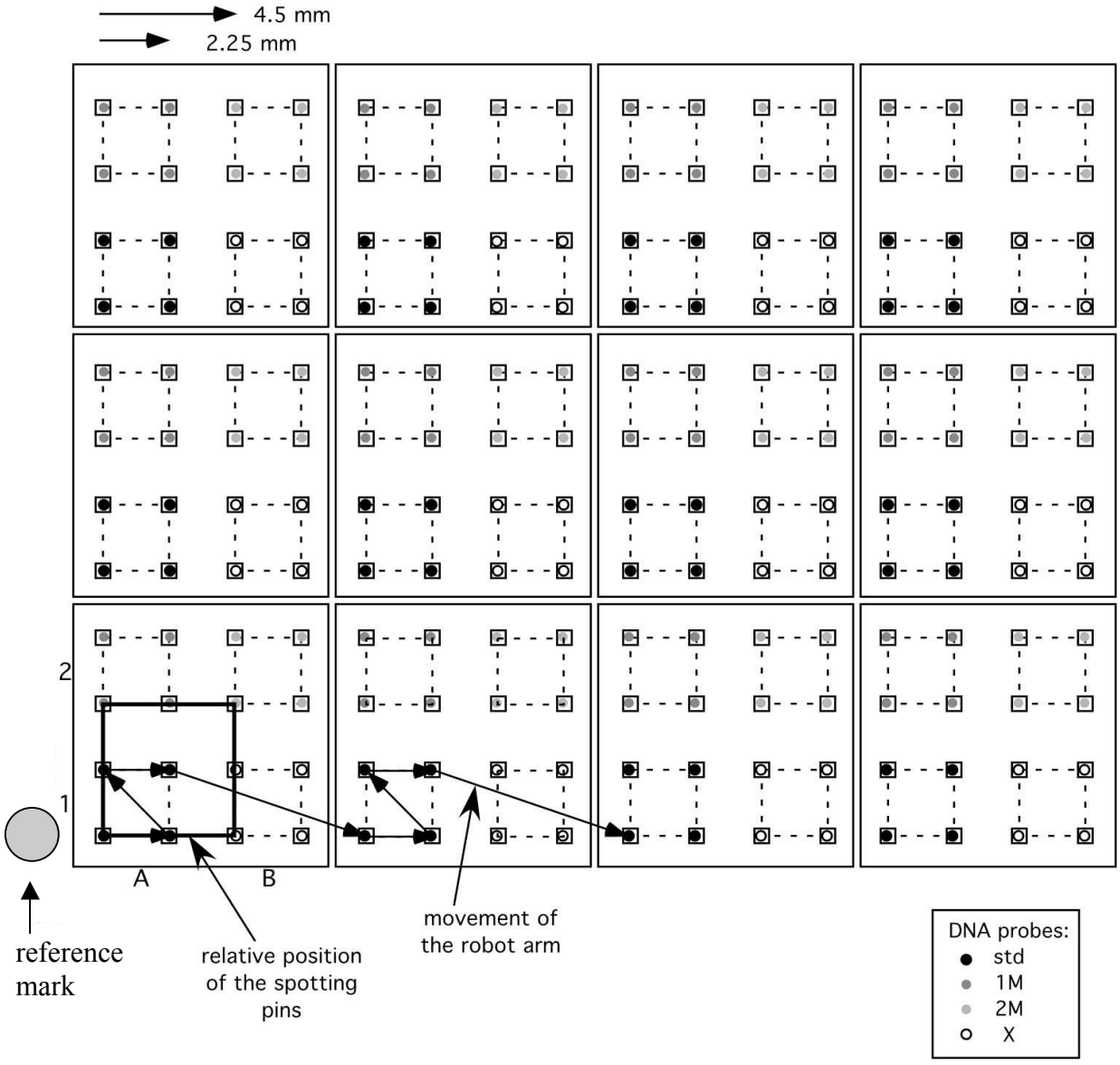
The fluorescence will be detected using a DNA array scanner:

DNA array Reader (Genewave)

We use a DiagArray™ which is a compact, versatile and intuitive fluorescence microarray reader designed for fast and accurate readout of slides. All specifications of the scanner are given on the product sheet (p.7).

Day 1:
SPECIFIC COVALENT BONDING OF DNA PROBE MOLECULES ON THE FUNCTIONALIZED GLASS SLIDES

In this part, specific DNA oligomers (probes) will be grafted on the Amplislide using a robot according to the scheme shown. Each robot pin will spot a defined DNA droplet. Four pins will be used and this process will be repeated over most of the slide surface.



Microarray Slides

ampliSLIDE™ 570/670

High Sensitivity Amino or Epoxy Coated Double Color Microarray Slides

16-FOLD ENHANCEMENT

ampliSLIDE™ technology from Genewave delivers the highest sensitivity for fluorescence based microarray applications.

ampliSLIDE™ incorporates the combination of a unique fluorescence-amplifying thin film optical coating and a high quality surface chemistry that provides the user with a large sensitivity budget.

Double Color **ampliSLIDE™** has been tailored to provide balanced fluorescence enhancement for Cy®3/Cy5 or analogous dyes.

Benefits

- *Significant improvement of microarray sensitivity*
- *Reliable detection of low-expressed genes*
- *Reduction of required samples amounts such as biopsies*
- *Reduction of the number of cell passaging steps*
- *Saving on costly reagents such as dye labeling amplification kits*
- *Faster microarray imaging*

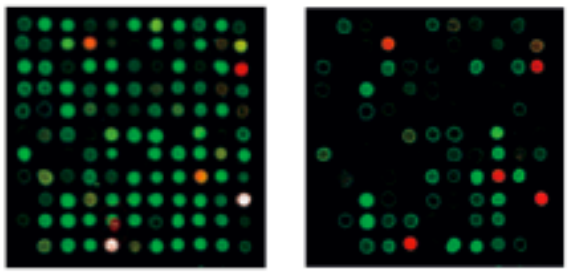
Key Features

- *10 to 20 fold signal enhancement*
- *Excellent probe binding efficiency*
- *Preservation of channel ratios*
- *Compatible with common microarray equipment and protocols*
- *Chemically robust reflective coating*
- *Easy identification of reflective side*

Significant signal enhancement

Fig. 1 - Comparison between identical high density DNA arrays spotted on AmpliSlide™ A and on a leading competitor's aminosilane slide.
These images show a very significant enhancement of the signal intensity on AmpliSlide™. Both slides were spotted, hybridized and scanned under the same conditions (including laser power and PMT gain value).

Data kindly provided by Dr. Nicolas Maunoury from CGM (Centre de Génétique Moléculaire, CNRS) - GDDMAP, Gif-sur-Yvette, France.



AmpliSlide™ | Conventional glass aminosilane slide

genewave

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AmpliSlide 570/670 A Substrates

Instruction Manual for DNA Microarray Experiments

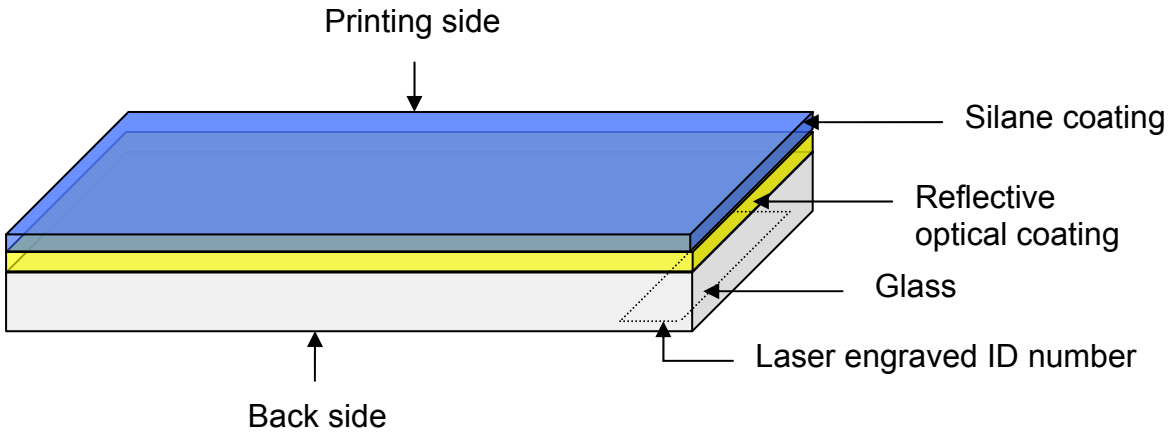
Introduction

Product Overview

AmpliSlide™ 570/670 A is a reflective aminosilane coated slide compatible with all standard microarray equipment, such as arrayers, hybridization stations and top-reading scanners.

Slides are produced from high quality glass to guarantee standard dimensions (75.0 x 25.0 x 1.0 mm) with excellent tolerances.

AmpliSlide™ 570/670 proprietary optical multilayer reflective coating is then deposited on the glass surface prior to chemical functionalization (see the scheme below). Constructive interference effects are exploited to enhance both excitation and emission of fluorescence. The emitted fluorescence is concentrated into a narrow cone and redirected onto the collection optics of the microarray scanner. This special reflective coating is finely tailored to provide up to 16-fold signal enhancement *balanced in both colors*, compared to conventional aminosilane glass microarray slides.



Scheme of AmpliSlide™ functionalized substrate.

The high quality and homogenous surface aminosilane coating allows efficient binding of amino-modified or non modified PCR products, cDNA and shorter probes (from 25 to 100 mers).

AmpliSlide™ 570/670 A substrates undergo strict quality controls at all steps of production to ensure our customers a product of high reproducibility and reliability.

Storage and handling

1. AmpliSlide™ 570/670 A can be stored at least nine months at room temperature (20 to 25°C) in its original and undamaged packaging. It must be used prior to the expiration date indicated on the package.
2. Slides must be used in a clean environment to avoid the presence of particles that can provoke defaults of printing or background noises when scanning.
3. If all slides contained in the package are not used at the same time, close cap as soon as possible after removing slides from container in order to maintain the cleanest environment as possible for the unused slides. Place the container back to its original pouch and dry store, for example in a desiccator, at room temperature and in dust-free and light-protected conditions.
4. In order to benefit from the high sensitivity conferred by the optical coating, take care of spotting AmpliSlide™ on the right side. Wrong side is easily recognizable thanks to the laser engraving (see instructions below).

General precautions

1. The protocols given in this document should be considered **only as technical guidelines** since some optimizations may be required depending on the application and array processing (printing technology, buffers available, nature of probes, hybridization tool…).
2. AmpliSlide™ 570/670 A is for research use only, not for *in vitro* diagnostic use.

Security Warning

1. Refer to manufacturers' supplied Material Safety Data Sheets (MSDS) for proper handling and disposal of all chemicals.
2. Beware of reflected laser light if using the AmpliSlide™ 570/670 A slides in an unshielded detection system (fluorescence microscope etc.)

Equipment required

- UV cross-linker or oven for probe immobilization
- Heated water bath or automated hybridization station
- Humidified hybridization chambers
- Compressed nitrogen gas or oil-free air or centrifuge equipped with slide holders
- Cover slips
- Coplin jars, slide staining rack or 50 ml tube for washings
- Stirring plate or orbital shaker

Reagents required

Reagents are not provided. This list is based upon the following advised protocol but may vary depending on experiment optimizations.

- Ultrapure water
- Dimethylsulfoxide (DMSO)
- Saline Sodium Citrate (SSC)
- Sodium dodecyl Sulfate (SDS) or sarkosyl
- Formamide
- Bovine Serum Albumine (BSA)
- Salmon sperm DNA or other nucleic acid blockers (i.e Calf thymus DNA)

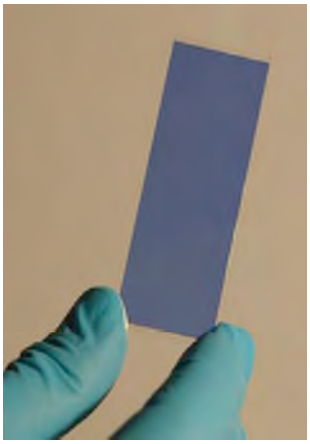
Printing

Spotter compatibility

AmpliSlide™ 570/670 A slides are compatible with all contact or non-contact arrayers.

Printing side

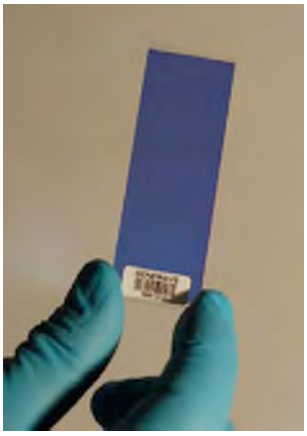
In order to benefit from the high sensitivity conferred by the high technology optical coating, take care of spotting AmpliSlide™ on the mirrored side.
Non-barcoded slides. The wrong side is easily recognizable thanks to laser engraving. Please make sure to array, hybridize and scan only the surface free of engraving. The "genewave" inscription and Genewave's traceability ID number ("GF033" on the picture below) should NOT be visible when you put the slides onto the spotter tray. In recent batches the inscription "wrong side" is added for even more easy identification (not shown below).



Correct (mirrored) side

Wrong (bare glass) side

Barcoded slides. The barcode label is applied on the mirrored (correct) side. Please make sure to array, hybridize and scan only the surface with a barcode label. These barcode labels should be visible when you put the slides onto the spotter tray.



Correct (mirrored) side

Wrong (bare glass) side

Printing area

The total area available for printing is 70x22 mm for slides without barcode and 63x22 mm for slides with barcode.

Silane activation (see chemical reaction scheme in appendix I)

Aldehydes combine with amines to form Schiff bases, which can be stabilized by oxidation. Glutaraldehyde is a bifunctional aldehyde that allows covalent coupling between γ-aminopropylsilane at the surface of the glass slide and amino-modified DNA oligomers deposited by the robot pins.

- 1rst treatment :

When purchased, Amplislides are vacuum sealed. This first treatment is only required when the slide remained several days in contact with ambient air. CO_2 contained in air reacts with terminal NH_2 and forms NH_2CO_3 , which prevents efficient coupling. This reaction is reversed by KOH treatment.

- Immerse the glass slide in a 0.1 M KOH aqueous solution during 15 min
- Rinse thoroughly with distilled water using the dispenser and dry with compressed air (be careful not to let the glass slide fly away !)

- 2nd treatment:

Be careful not to breathe toxic glutaraldehyde fumes. Work in the fume hood, wear gloves and a labcoat.

- Immerse the glass slide in a 10% glutaraldehyde (Sigma G-7651) aqueous solution and gently agitate during 45 min at room temperature (rotamax).
- Rinse with distilled water using the dispenser and dry with compressed air.

DNA probe grafting using the MicroGrid robot (BioRobotics)

Four DNA oligomers are used :

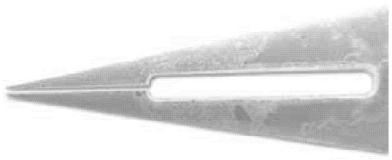
- a "standard" sequence : "Std" ⟶ TTTTTGATAAACCCACTCTA
- a standard sequence with one mismatch: "1M" ⟶ TTTTTGATAAA**G**CCACTCTA
- a standard sequence with two mismatches: "2M" ⟶ TTTTTGATAAA**GA**CACTCTA
- an "unrelated" sequence "X" ⟶ TTTTTTTTTCCAAGAAAGGACCCG

These oligomers are covalently modified at the 5'-terminus by an amine (Apibio, Grenoble). The DNA concentration in the stock solution is 200 μM.
Prepare a 50 μL solution of the different probes at 10 μM in 0.3 M sodium phosphate solution and transfer in the 384-well sample plate of the robot.

The four probes will be spotted onto the glass slide using a robotic arrayer.
The defined parameters of the robot are: the type of sample (silicon wafers or glass plates), the number of probes, the number of pins used, their type, the spotting velocity, the basic motif and the number of repeats, the washing procedures. The slide is held on the robot moving plate by vacuum suction. A reference mark will be drawn near the leftmost bottom spot (A1).

Two types of pins can be used: solid pins and quill pins that deliver 250 μm and 180 μm spots respectively. We are going to use quill pins.

solid pin



quill pin

When all parameters have been defined, the spotting procedure is launched. The slides are then incubated **overnight** at room temperature in a humid atmosphere.
The next morning, a stabilization treatment is performed by the CIME technical staff.

- $NaBH_4$ reduction of pending aldehyde bonds (CHO → CH_2-OH) and N=C stabilization.
- Stringent washing with an anionic detergent (0.2% SDS) to remove the probes that are not covalently bound to the slide.

The DNA chips are then rinsed with distillated water and dried with compressed air. They can be stored at 4°C under nitrogen gas and are stable for several months.

DAY 2 :
HYBRIDIZATION AND QUANTITATIVE FLUORESCENT DETECTION OF TARGET DNA ON THE GLASS SLIDES

The DNA chips are now ready for use. Hybridization will be conducted with a fluorescently labeled DNA solution (target), and quantification of bound target DNA will be performed.

Set the thermostat oven to 42°C. Prepare wet chambers: moistened paper in tightly closed plastic boxes.

I – HYBRIDIZATION (see appendix II)

The target DNA sequence is: 5'-CATAGAGTGGGTTTATCCA-3'

This oligomer is covalently modified at the 5'-terminus by a Cy3 fluorophore (Apibio, Grenoble). The DNA concentration in the stock solution is 200 μM.
Prepare a 50 μL solution of the target DNA at 0.2 μM in 1x hybridization solution (Sigma H-7140). Spread the solution evenly over the slide and cover with Parafilm. Put the DNA biochip in a wet chamber and incubate for 45 minutes at 42°C.

Note that we use single strand DNA. For double-strand DNA, a denaturation step is necessary before hybridization (3 min, 90°C).

Prepare the wash solutions of increasing stringency: 2x SSC and 0.2x SSC

1st wash:
Put the DNA chips in a Petri dish and add the 2X SSC solution. The Parafilm is released and floats on top of the solution. Wash during 2 min on the Rotamax at 50 rpm.

2nd wash:
Wash once in 0.2x SSC. Dry the slides over a piece of absorbing paper and remove droplets with compressed air.

Store in the dark.

II – FLUORESCENCE DETECTION AND ANALYSIS

This part consists in detecting and quantifying the fluorescence associated with the different DNA probes on the glass slides.
We will use a laser scanner capable of exciting both Cy3 and Cy5 fluorophores; the detection will be done with a 9μm, 16 bit CCD camera. The fluorescence readout will be given in image files showing the array of DNA hybridization spots and quantitative data analysis is provided by the software and exportable as an xls file.

NEW

ampliREADER™

Compact CCD-based Microarray Reader

LOW COST

While tackling the long standing issue of costly microarray scanning, Genewave™ developed *ampliREADER™* which is built upon a novel lightweight optical architecture, providing state-of-the-art performance at a very attractive price. *ampliREADER™* comes with the Array4D™ microarray analysis matching software package, a tool designed by and for microarray users. *ampliREADER™* is suited for all biochip applications, be it differential gene expression studies, genotyping, polymorphism detection, sequence identification or protein arrays.

Benefits

- *Easily affordable double-color microarray reader*
- *Perfect match with AmpliSlide™ substrates*
- *Powerful and intuitive microarray software for increased workflow*
- *High dynamic range that allows various applications from fast diagnostics to gene expression studies*
- *Extra-small foot print: only 26 x 35 cm (10 x 14 inches)*

Key features

- *Original wide-field optical architecture*
- *Fast acquisition time*
- *Dye compatibility: Cy®3 & Cy®5 and analogous*
- *High quantum efficiency cooled CCD*
- *Integrated barcode reader*
- *OEM version and other wavelengths on demand*

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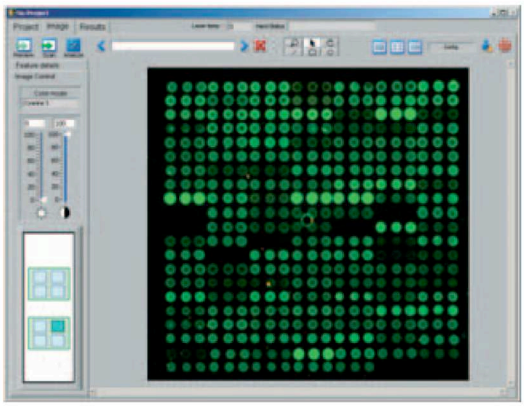
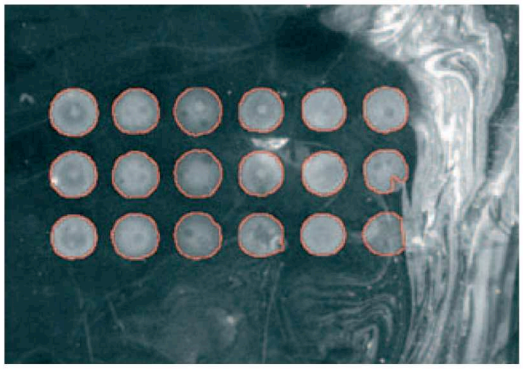
Innovative microarray analysis software

Array4D™ is the microarray analysis software solution from Genewave, the perfect partner to AmpliReader™. Array4D™ focuses on ease of use and efficiency. It is fast and intuitive in both routine and advanced uses, thanks to innovative features such as its level navigator and advanced viewing modes.

Array4D™ was designed by and for day-to-day microarray users.



Advanced feature extraction engine



Array4D™ is always on the spot thanks to SureGrid™ and SureSpot™ algorithms even with less than perfect microarrays

Under the hood lies a fast, robust and powerful image analysis engine, comprised of SureGrid™ gridding and SureSpot™ segmentation.

SureGrid™ precisely aligns the grid with the image, using an automatic procedure. 'SureSpot' finds the spot contours and associated background regions, with an outstanding behaviour with respect to stains and other artifacts that might lead other algorithms into error.

In practice, this combination of superior technologies means you can drastically reduce the time you spend checking the validity of your data before actually interpreting it.

Product specifications

Excitation wavelengths	535 nm and 640 nm
Dye compatibility	dyes emitting at around 570 and 670 nm
	• CY®3 and Cy®5
	• Alexa®546 and Alexa®647
	• DY®-548 and DY®-648
Sample format	standard microscope slides
Maximum imaged area	22 x 72 mm
Typical acquisition time	3 min per slide
Pixel size	15 to 60 µm
Detectivity	1 fluor/ µm²
Pluconnectivity	standard USB/Ethernet
WCight	14 kg (30 lbs)
Dimensions	35 x 26 x 45cm (14 x 10 x 18 inches)
Power supply	90-260 VAC, 50 W
Barcode Reader	all formats
Microarray analysis software	Array4D™ included
Data format	16 bit tiff
Hardware requirements	2GHz processor, 1Gb RAM, Windows XP SP2

Ordering information

Product	Designation
4AR4600-L-535-640	AmpliReader™ 4600, 535/640 nm ; Array4D™

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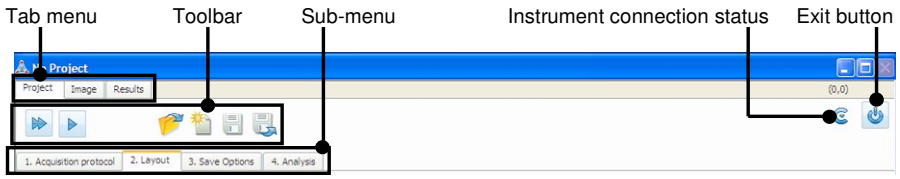
PS004-05_2007-11

The Array4D™ Main Window

Click the Array4D™ icon on the PC desktop to launch the software. Array4D™ software user interface is then automatically displayed on your PC screen. This is the starting point for sending commands, entering information, or receiving status information. This is also where scanning images and quantification results are displayed.



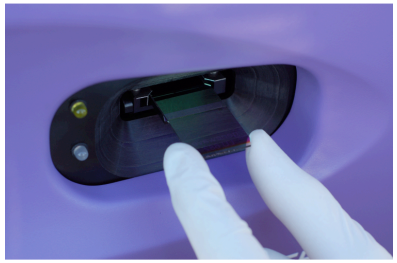
The main functions are:

- A Tab-menu with three tabs, namely Project, Image and Results.
- Sub-menus sections for each menu.
- An instrument connection status icon indicating whether the Amplireader™ scanner is connected or not to your PC.
- The Exit button.

Scanning a microarray

To load a microarray slide:

1. Hold the slide by the edges
2. Hold the slide with arrayed features facing up, and with the barcode or label towards you. Gently insert the slide into the main slot and push it until it is fully inserted.

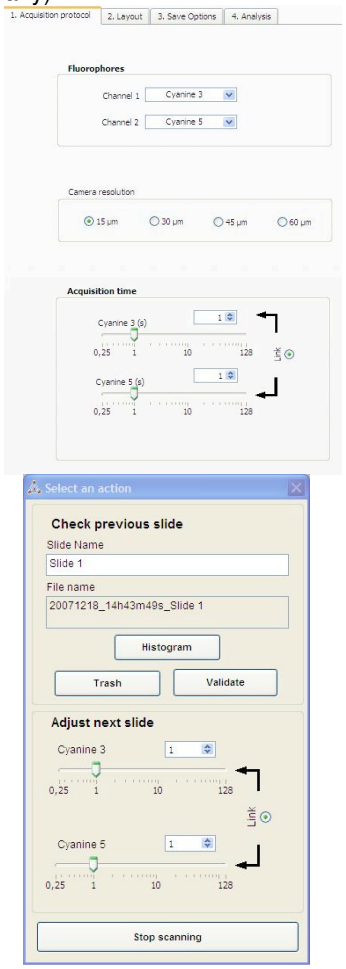


Preview

The Preview scan is a fast low-resolution scan that will help you find the area on the array that you want to scan at higher resolution for further analysis. The image will not be saved.

Scan Area

The **ROI** icon on the Image toolbar allows you to scan at a 15-µm resolution a selected area (minimum size 3-by-3 millimeters). Select for example a region that covers one third of the slide area (button). You must then set the other acquisition parameters in the **Project** / **Acquisition Protocol** sub-menu, i.e. the fluorophore colors (select Cyanine 3 and Cyanine 5 for ex.) and an acquisition (exposure) time for the color(s) selected (choose any).



Press the **Scan ROI** button**.** Amplireader™ acquires sets of images from the slide and automatically ejects the slide when finished.

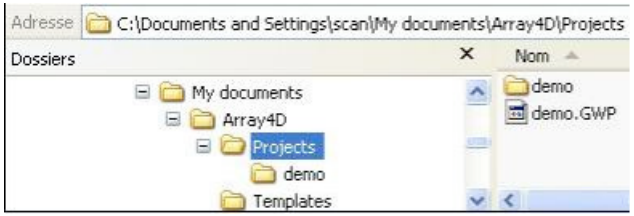
Saving the Images, Opening a Project

At the end of the scan, a **validation** window will automatically open up.

- Change the slide name from “slide1” to “demo1”
- Click **validate** to save the scan.

Since the project was not created before scanning, a window opens up to allow you to choose the name and the save location of your project.

- Save the project under the filename “demo” and validate. The “demo.GWP” file contains all the settings parameters of the project; images are to be found in the “demo” folder.



Viewing the Images

Scanned images are displayed under the ***Image menu***. The **Image toolbar** gives access to scan and analysis icons, image navigator and statistics tools, slide navigation control as well as layout and segmentation views.

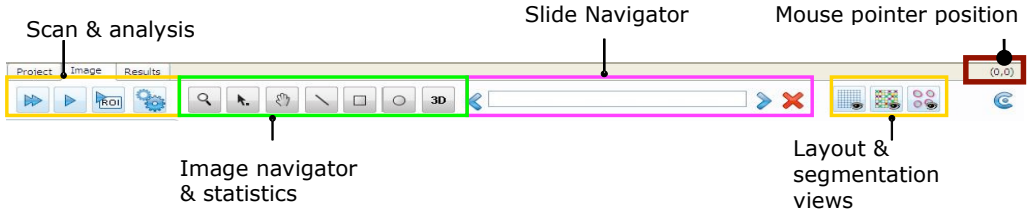


Image navigator and statistics toolbox

Zoom: left click to zoom in, shift + left click to zoom out

Pan: moves the scanned image on the screen manually

Default: mouse pointer information in the *feature details* panel

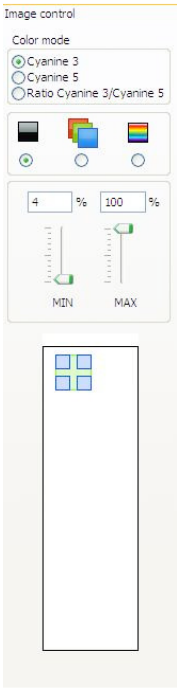
Line profile: pixels intensity profile and statistics along a line

Rectangular region histogram: pixel statistics in a rectangular area

Elliptical region histogram: pixel statistics in an elliptical area

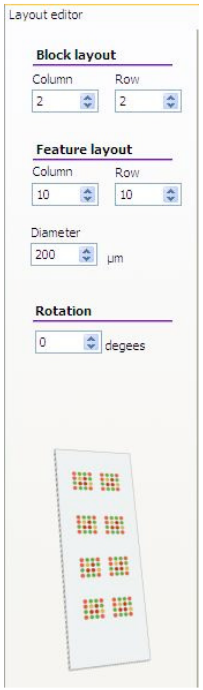
3D Tool: 3D profile of the selected area

Image panel sub-menus:



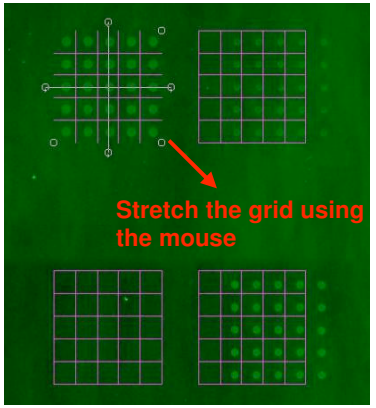
In the **Image Control** submenu, use MIN, MAX cursors to enhance the image display (from the default position, decrease the MAX cursor until the spots are clearly visible).
You can also select the color mode and choose the colormap (gray scale, single color or rainbow).

The second **Feature details** sub-menu allows you to view detailed views and pixel values as the mouse cursor moves over specific features. The position of the cursor can be read on the Image toolbar.



Analyzing the Images

How to adjust a layout?

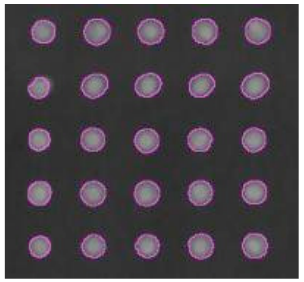


Select the sub-menu **Layout editor** on the **Image menu**. A default pattern of 4 **grids** is automatically created on the upper left side of the image.
1. The first step consists in defining the block layout (rows and columns), the feature layout (rows and columns) and the feature diameter (all dimensions are in microns).
2. Using the mouse, move and resize the grid until it fits on the image. Note that the mouse cursor changes shape depending on its position on the block:

Usually, it is best to place the upper left corner of the grid on the upper left spot and then to resize the grid until the lower right corner fits to the lower right spot. Eventually, each spot should fit within one square of the grid.
A **rotation tool** can help you to get a finer adjustment in case the spotting layout falls out of the slide axis.
Pitches and **Offset** are automatically displayed under the Project Layout sub-section tab (press the Project tab and select Layout tab to view the values of adjusted pitches and offset).


Press the **analyze** button to launch the quantification.

Viewing the Quantification Results


Segmentation view

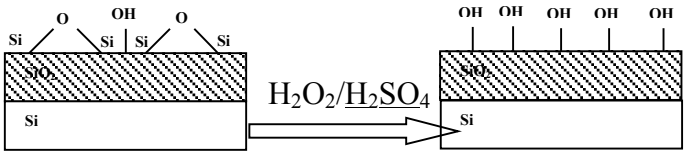

Click the **view segmentation** button to display the results of the segmentation on the image.


Click the **view grid layout** button to view the adjusted grid on the image.

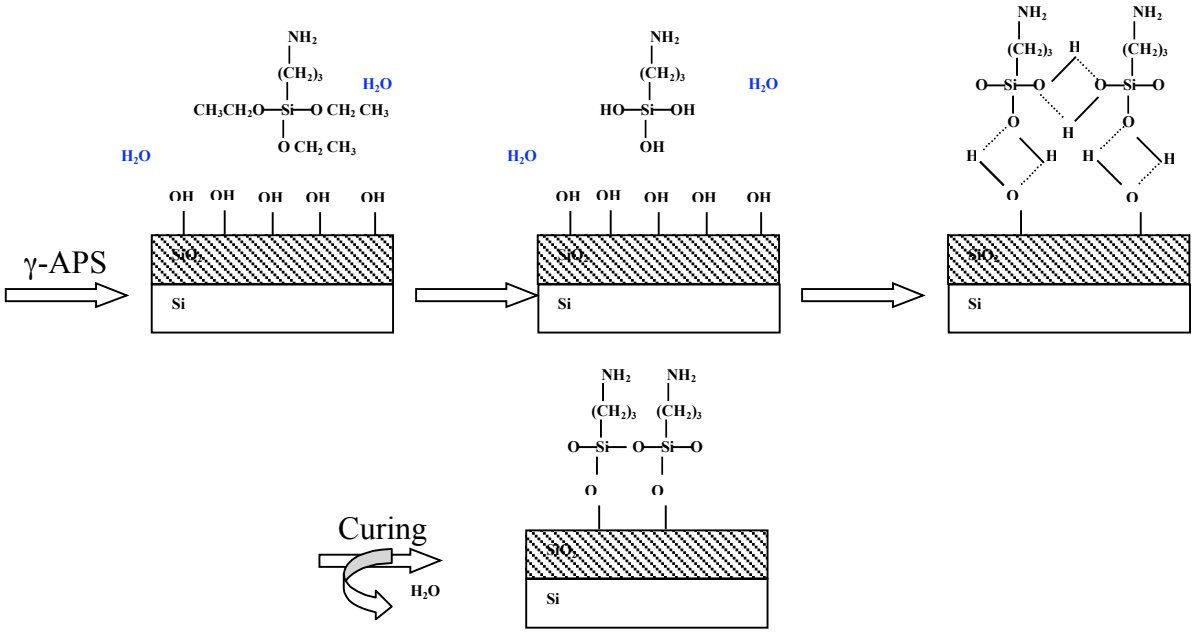
Following image analysis, quantification results are displayed as a spreadsheet in the **Results menu**. A number of fluorescence intensity statistics are available (mean, median, standard deviation, etc) for each individual spot.
Results are automatically saved in the project file “demo” together with the images. They are exported as **gpr** files.

APPENDIX I : CHEMICAL COUPLING OF DNA PROBES TO A SILICON OXIDE SUBSTRATE (OR GLASS)

HYDROXYLATION

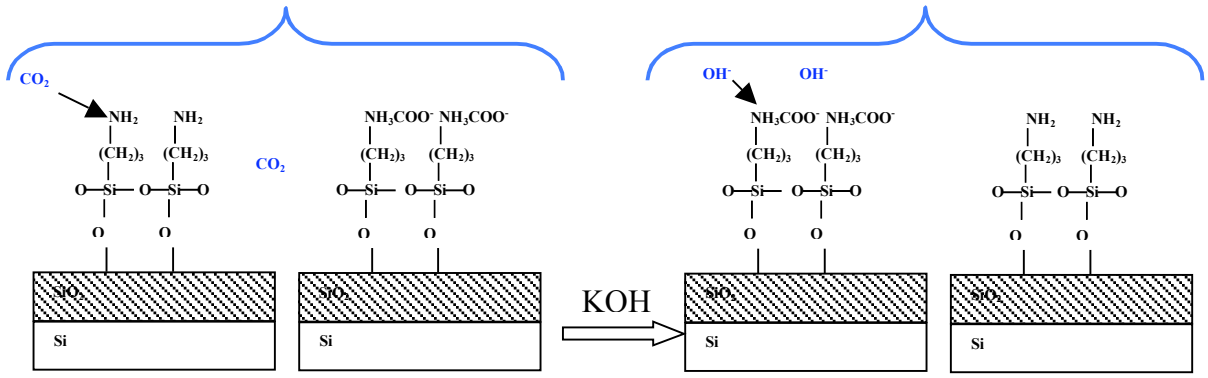


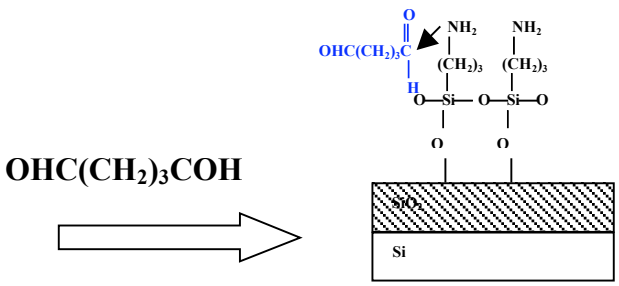
SILANIZATION



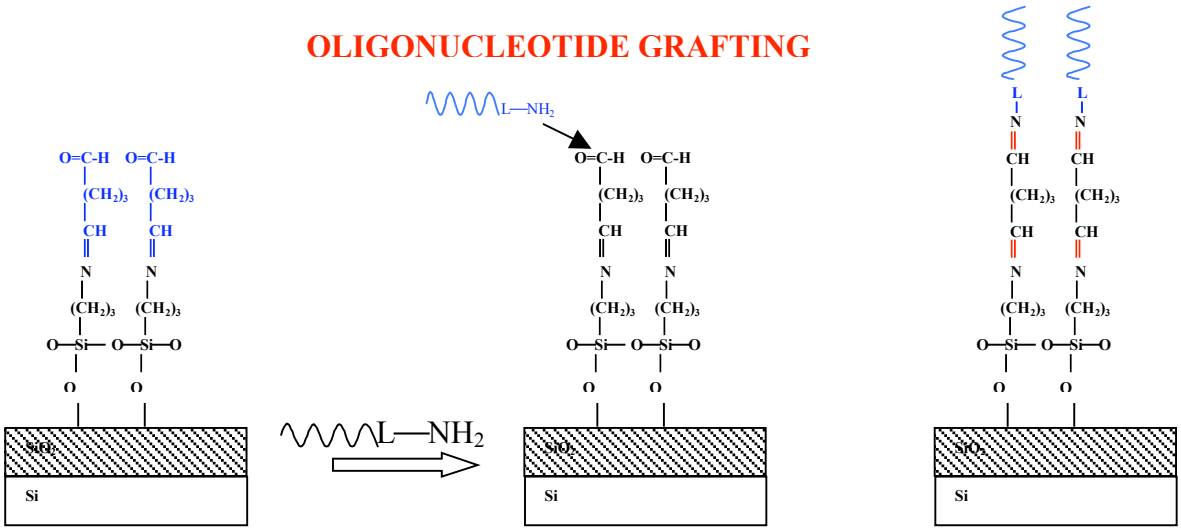
Atmospheric CO2 INACTIVATION

RE-ACTIVATION

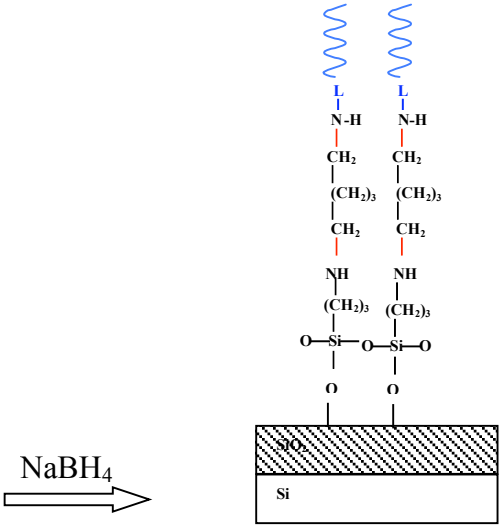




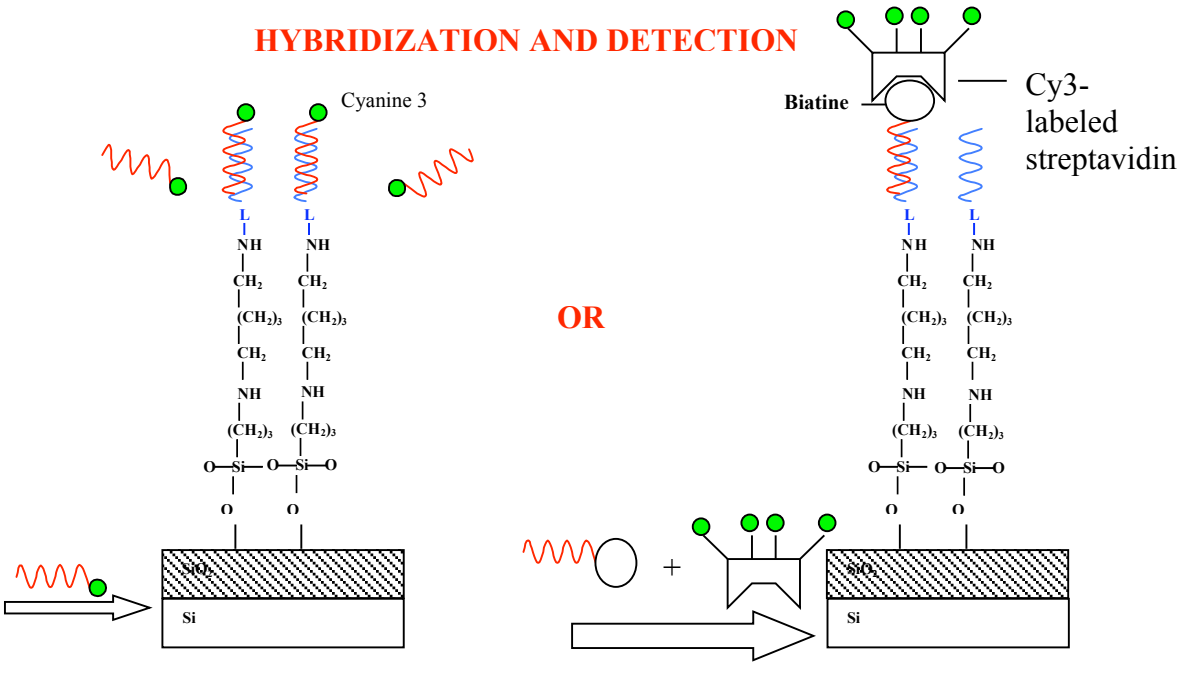
OLIGONUCLEOTIDE GRAFTING



STABILIZATION TREATMENT

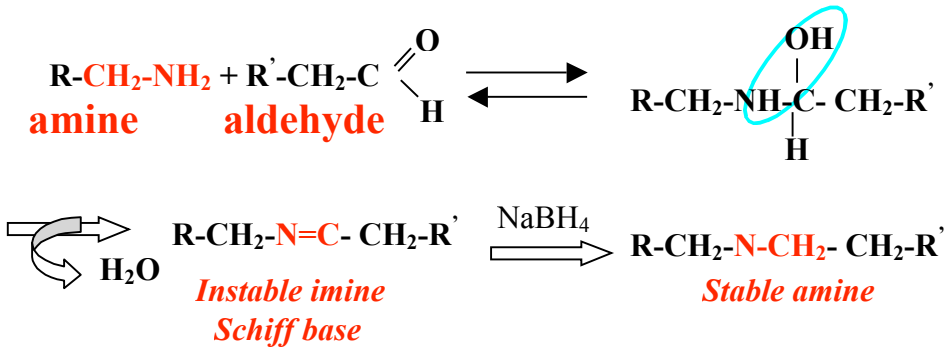


HYBRIDIZATION AND DETECTION



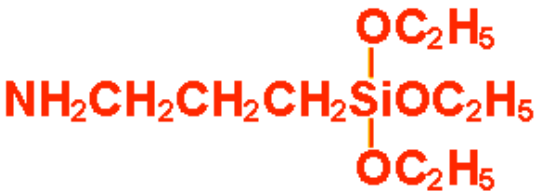
Hydroxylation creates OH bonds on the silicon oxide surface that react with the silane group of γ-APS (3 aminopropyl triethoxysilane) in the silanization process. Curing stabilizes the aminopropyl silane layer by converting hydrogen bonds to a covalent Si-O-Si network. During prolonged storage, the bonding capacity of the surface decreases because of:

1) NH_2 instability, which reacts with atmospheric CO_2 to form $NH_3^+COO^-$. The amine group is restored by the KOH treatment.
2) O-Si-O bond instability releasing silane.



Silane chemistry and applications
(from the Dow Corning silane home page : http://www.dowcorning.com/content/silanes/)

Silanes are monomeric silicon compounds with four substituent groups attached to the silicon atom. These substituent groups are generally a combination of inorganically reactive and organically reactive groups.



γ-aminopropyltriethoxysilane (APTES)

Inorganic reactivity represents the covalent bonds formed through oxygen to the silicon atom to form a siloxane type of bond. This allows silane coupling to other silicon atoms.

Organic reactivity occurs on the organic portion of the molecule and does not directly involve the silicon atom. The reactivity of organic groups attached to silicon is similar to organic analogs in carbon chemistry.

This large number of possible combinations explains silicon's versatility and its ability to be used in a variety of ways with carbon-based chemicals. The basic structure of organofunctional silanes is: $R_nSi(OR)_{4-n}$ (with "R" being an alkyl, aryl, or organofunctional group and with "OR" being methoxy, ethoxy, or acetoxy). The OR group is readily released by hydrolysis, for instance in aqueous solutions.

APPENDIX I: HYBRIDIZATION

The strands of DNA molecules are held by non-covalent interactions, which are disrupted by modifying the physico-chemical conditions (**denaturation**). This process is fully reversible (**renaturation**). Considering a given DNA single strand, nucleic acid strands of any origin are able to associate with it, provided that some complementarity exists between the two molecules: this interaction is called **hybridization**.

Many molecular techniques rely on nucleic acid hybridization: polymerase chain reaction (PCR), electrophoresis and gel blotting techniques (Southern and Northern blots), microscopy localization of genes on entire chromosomes (FISH) etc …

Essentially, DNA biochips are developed for four purposes:

- Analysis of single gene allele variations in individuals, to help defining molecular medical techniques.
- Species identification (for instance pathogens).
- Gene expression studies, to determine gene usage as a function of the cell, organism, population environmental conditions.
- Gene editing studies, to measure the expression of mRNA alternative splice variants.

PRINCIPLES OF HYBRIDIZATION :

DNA molecules can be hybridized to other DNA or RNA molecules. DNA is generally double strand and RNA single strand. In DNA chips, **probes** are either large DNA molecules such as PCR products, or small short single strand sequences obtained by chemical synthesis called **oligomers**. The nucleic acids to be tested (**target**) usually are DNA fragments, a DNA copy of expressed RNA (cDNA) or RNA. The target molecules are generally fluorescently labeled, since direct detection of DNA hybridization is so far not efficient enough to analyze tiny DNA amounts.

The molecular basis of this association is the formation of hydrogen bonds between complementary bases A and T, C and G. In addition, the two strands should run in opposite directions (5'→ 3' ends). Since A::T and G:::C pairing involves 2 and 3 hydrogen bonds respectively, the stability of nucleic acid complexes depends on the GC content of the sequence involved in their binding. It should be noted that the association between two nucleic acid molecules does not require perfect complementarity and the presence of **mismatches** simply decreases the total binding energy.

The presence of similar gene products in genomes or of different gene alleles in populations makes it necessary to discriminate between close sequences. Therefore, the physico-chemical conditions of the hybridization (**hybridization stringency**) need to be adjusted to render the assay sensitive enough, ultimately allowing single base differences.

CONTROL OF HYBRIDIZATION STRINGENCY :

Three parameters need to be taken into consideration in hybridization experiments:

Temperature

The association of two nucleic acid strands results from the balance between hydrogen bonding, that favors the association, and thermal agitation that disrupts them. As a result, the association between target and probe depends on the temperature (Fig. 1 left).

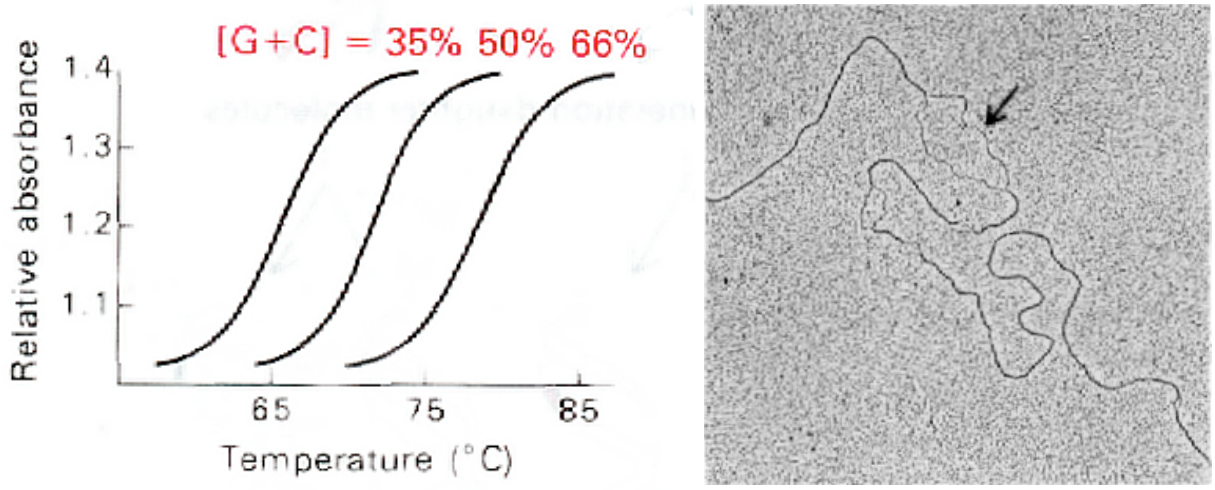


Fig. 1: left: DNA melting temperature at three GC contents (hypochromic effect); right: localized alkaline DNA denaturation in AT-rich regions (TEM observation)

This curve is called a melting curve and the melting temperature T_m is the temperature at which half of the target DNA is bound to a probe DNA. The melting temperature depends on the GC content of the pairing strands, the number of mismatches, and the physico-chemical conditions. For short oligonucleotides, predictions about T_m can be obtained at the following websites:

http://www.microbiology.adelaide.edu.au/learn/oligcalc.htm

http://alces.med.umn.edu/rawtm.html

In the case of the DNA "standard" probe and "target" oligonucleotides used in this lab work, the T_m is estimated to 42°C (using the pairing sequence : GATAAACCCACTCTA and 150 mM salt).

The presence of one mismatch decreases Tm by 1.5 or 10 °C, for 100 or 20 bp oligonucleotides, respectively. For larger DNA molecules, a commonly used formula is that of Bolton and MacCarthy (1962):

$$T_m = 81.5 + 16.6 \log_{10} [Na^+] + 0.41 (\% \text{ of } G + C) - 600/N - 0.65 (\% \text{ of formamide})$$

where N is the nucleotide number.

This equation only works for Na+ concentrations of 1 M or less. It has been shown experimentally to be valid for sequences as long as 60-70 nucleotides and as short as 14 nucleotides. For large DNA molecules, melting occurs in separate domains according to the local GC content. Alternatively, the T_m temperature can be experimentally determined using the hypochromic effect of DNA renaturation.

Hybridization experiments are usually carried out at a temperature 5°C below the T_m for small oligonucleotides and 25°C below the T_m for larger DNA probes.

Physico-chemical conditions

Various parameters influence strand pairing.

pH: under alkaline conditions, the phosphate groups on the DNA strand backbone are charged, resulting in strand separation. Note that alkaline denaturation is not used for RNA molecules, since this also favors hydrolysis of the phosphodiester bonds along the strand.

Ion concentration (ionic strength): at neutral pH, negative charges are present on the phosphate groups along the DNA strand backbone. The concentration of counter-ions in the solution therefore modulates the electrostatic repulsion between DNA strands.

Hydrophobic interaction: during pairing, DNA bases stack together, and some binding energy is gained through the interaction between the π-electrons of the pyrimidine and purine rings. This creates a somehow hydrophobic environment, where non-polar molecules can accumulate. Conversely, addition of detergents weakens the stability of DNA pairing.

Denaturating agents: when using mRNA or cDNA targets, formamide is often used as denaturing agent since it allows lowering temperature without losing specificity. Furthermore, 50% or greater formamide favor DNA-RNA hybridization over DNA-DNA hybridization.

High molecular weight polymers (Ficoll, PVP): during hybridization, they increase the effective nucleic acid concentration by excluding volume from the hybridization mixture.

Concentration and time

It should be noted that DNA hybridization is essentially a non-equilibrium technique. These techniques use very small amounts of probe and target molecules. Furthermore, probes are immobilized on a substrate and the bulk target DNA concentration is generally much lower than the local probe concentration. Thus, one difficult point is to put target DNA in contact with probe DNA molecules.

Experimentally, after denaturation, target DNA is incubated on DNA chips for a relatively long time, at a temperature lower than the T_m and at rather low stringency, in order to favor the association between target and probes. Then, washings are performed rapidly, at room temperature, in more stringent conditions, in order to remove loosely bound molecules while keeping as much as possible specific target-probe interaction intact. Longer washing times will increase the specificity, but at the expense of a decrease in fluorescent signal. This explaines why washing procedures are explained in details.

DETECTION OF HYBRIDIZATION :

When labeled DNA or RNA is used, the fluorescence intensity is scanned over each spot. The same DNA sequence is immobilized on several spots (usually 3), in order to determine statistical variations. For quantification of gene expression, each gene sequence is represented by different oligonucleotide sequences, in order to discriminate between similar sequences (overrepresentation). Therefore, most DNA chips display more than 100 different spots, up to 20000. Data representation and handling becomes then a major issue in the efficient use of the technique.

COMPOSITION OF STANDARD HYBRIDIZATION AND WASHING SOLUTIONS :

Control of the pH and ionic conditions is usually achieved by using a dilution of a concentrated stock, such as the 20 x SSC or 20 x SSPE solution:

20 x SSC (Sodium Salt Citrate)
for 1 Liter
175.3 g sodium chloride
88.2 g sodium citrate
adjust the to pH 7.0 with NaOH
sterilize
1x SSC is 0.15 M NaCl and 0.015 M sodium citrate at pH 7.2-7.4

20 x SSPE (Soldium Salt Phosphate EDTA)
for 1 Liter
175.3 g sodium chloride
27.6 g NaH2PO4-H20
7.4 g EGTA
adjust the to pH 7.0 with NaOH
sterilize

Prevention of DNA non-specific binding to surfaces (such as plastics, glass, or silicon) and acceleration of hybridization by excluded volume effect is achieved by adding a polymer solution called the Denhardt's reagent and an excess of an unlabeled DNA solution (usually denaturated fragmented salmon or herring sperm DNA)

Denhardt's reagent
For 1 Liter
10 g Ficoll (type 400)
10 g polyvinylpyrrolidone
10 g Bovine Serum Albumin (fraction V)
Filter sterilize

Hybridization solutions are made by diluting an unlabeled DNA solution in 5x to 6x SSC or SSPE and 1x to 5 x Denhardt's solutions. The composition of the hybridization solution used in the lab work is given:

Sigma H-7140 hybridization solution (1 x)
100 μg/mL DNA
5 x SSC
1 x Denhardt's
Filter to 0.2 μm

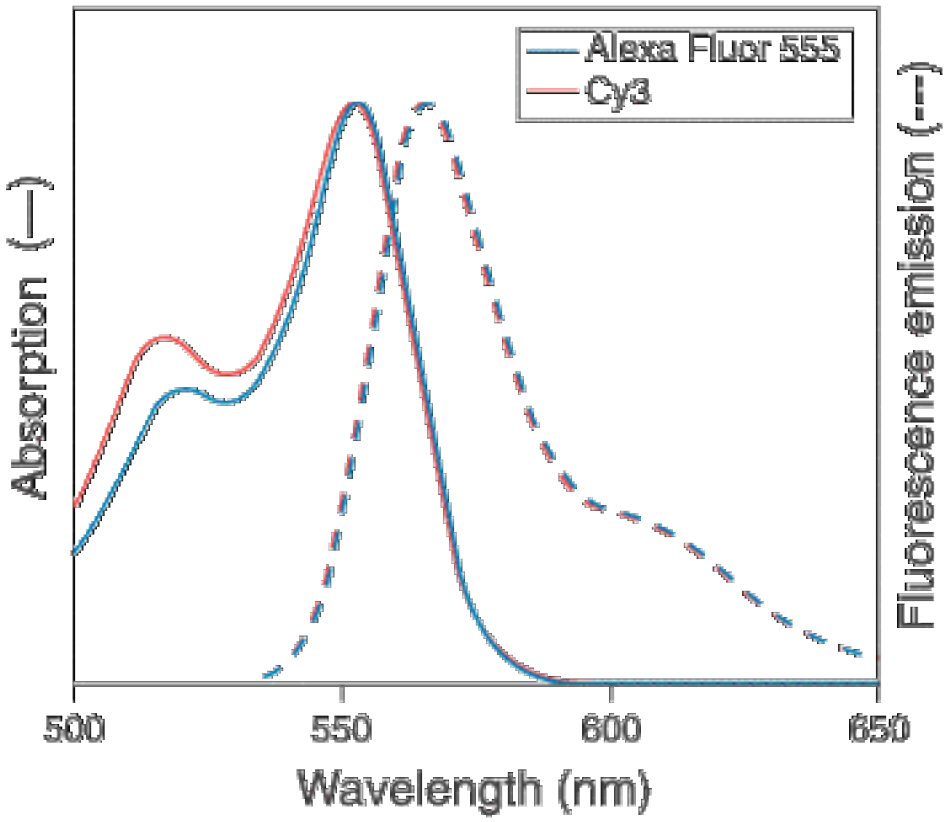
Here are two examples of washing conditions:

Example 1
2 x SSC, SDS 0.1% 5 min x 2
2 x SSC, 5 min x 2

Example 2
2 x SSC, 2 min
0.2 x SSC, 2 min

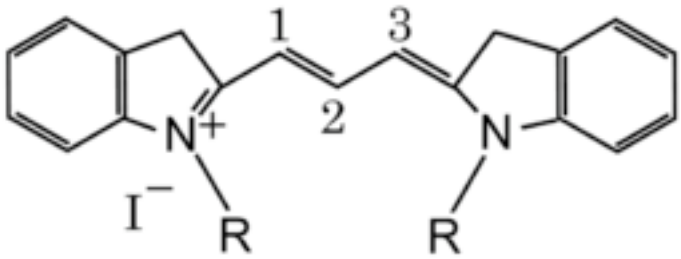
APPENDIX III : FLUORESCENT DETECTION OF CY3-LABELED DNA

Absorption and emission spectra of Cy3 dye

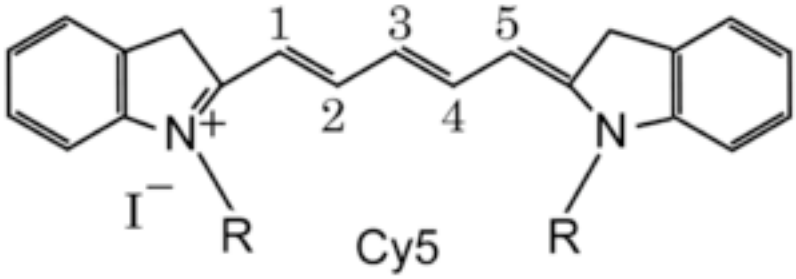


Cyanine 3 and Cyanine 5 fluorescent chromophores

Cy3 is excited maximally at 550nm and emits maximally at 570 nm, in the red part of the spectrum; quantum yield is 0.15; FW=766.
Cy5 is excited maximally at 649 nm and emits maximally at 670 nm, in the far red part of the spectrum; quantum yield is 0.28. FW=792.
The R-group is used to attach these chromophores to molecules of interest, e.g. target DNA.



Cy3



Cy5

TRACKING SHEET

The production and use of a DNA biochip involves multiple steps. In order to track down flaws in the operating procedures, it is advisable to note any changes made to the written protocol or any observations made. Please note here: