“BioChips ”: DNA and Protein Microarray Technology: fabrication and applications

This practical work consists of 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** (8-17 h) – DNA microarray: DNA will be extracted from a bacterial sample, amplified, fluorescently labelled and hybridized overnight to an oligonucleotide microarray.

**Day 2** (8-17 h) – DNA microarray: after washing, the fluorescence associated with each oligonucleotide probe will be detected and analyzed.

– Protein microarray: SPRi analysis of the interaction of antibodies with antigenic peptides covalently grafted to a prism. Interaction specificity and affinity will be determined.

This lab work was prepared by Franz Bruckert, Marianne Weidenhaupt, Didier Delabouglise, Laurent Nault, Claire Nicolas and Fabien Dalonneau at the Grenoble Institute of Technology.
Activity at a glance

Goal:
Introduction to microarray hybridization techniques, focusing on DNA extraction, labeling and hybridization conditions. These physico-chemical techniques are common to microarray technology for nucleic acid analysis.

Demonstrate Surface Plasmon Resonance (SPR), a biophysical method to investigate protein interactions. This sensitive technology is commonly used for affinity measurements and has the advantage of being feasible without protein labelling and purification.

Learning objectives:
Upon completion of this activity, students will be able to:
• Isolate plasmid DNA from bacteria,
• Fluorescently label a PCR product,
• Hybridize to an oligonucleotide microarray prepared in advance,
• Measure the fluorescence associated with each probe with a microarray scanner.
• Use SPR in the imaging mode to demonstrate antigen-antibody (Ag-Ab) specificity
• Deduce affinity of Ag-Ab interaction from SPR association/dissociation curves

During waiting periods, microarraying will be demonstrated.

Prerequisite skills:
• Prior practice with micropipettes.
• Basic knowledge of DNA structure and techniques (extraction, precipitation, PCR)
• Basic knowledge of protein structure (especially antibodies) and interaction

Flowchart and timeline:

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[DAY 1]
Check DNA prep by agarose gel electrophoresis ⇐
Preparation of ULS labeling reagent ⇒

Bacteria
Plasmid DNA extraction (optional)
Amplification of a DNA fragment of interest by PCR
DNA precipitation
DNA denaturation
DNA labeling
Purification of labeled DNA on gel-filtration spin column
Preparation of target DNA solution

Prepared in advance
Glutaraldehyde treatment of APTES-treated glass plates
Microarraying DNA probes
Stabilizing DNA probes with NaBH₄ reduction
Pre-hybridization treatment
```
continued from day 1
Overnight hybridization (T = 55 °C)
↓
Stringency washes
↓
Fluorescence analysis

DAY 2

**Antibody-antigen interaction measured by SPRi**

- Determination of resonance angle
- Calibration of the SPR signal on the prism
- Injection of different antibody solutions
- Acquisition and analysis of association and dissociation kinetics

Prepared in advance

- Glutaraldehyde treatment of NH₂silane-grafted gold prism
- Microarraying of peptides
- Stabilizing peptides with NaBH₄ reduction
SUMMARY

Activity at a glance
Summary
Introduction
Step 1 : Plasmid DNA purification using the QIAprep spin miniprep kit
Step 2 : Amplification of a DNA fragment of interest by PCR
Step 3 : DNA labeling
Step 4 : DNA hybridization with the oligonucleotide microarray
Step 5 : Fluorescence scanning
Appendix 1 : Primer and oligonucleotide sequences
Appendix 2 : DNA labeling chemistry
Appendix 3 : Cy3 and Cy5 fluorescent chromophores
Appendix 4 : DNA probe microarraying
Appendix 5 : Probe immobilization chemistry
Appendix 6 : DNA hybridization
Materials needed and ordering references
MicroGridII spotting robot operation and screenshots of parameter menus
Introduction

DNA microarray technology has become important to analyze the genome (comparative genome hybridization, single nucleotide polymorphism analysis, pathogen identification), the transcriptome (gene expression profiling, alternative splicing analysis, microRNA analysis) and even DNA-protein interactions (protein binding sites on DNA: Chromatin immunoprecipitation chip, DNA methylation mapping: Methyl-DNA immunoprecipitation chip, DNA regulatory elements: DNaseI chip). In this practical, we are going to label a DNA sequence of interest and hybridize it with a simple spotted oligonucleotide microarray. The use of a microarray fluorescence scanner and of a spotting robot will be demonstrated.

The practical demonstrates that DNA microarray can be used to identify the plasmid carried in a bacterial strain. After purification (1), the region of interest of the plasmid (insert) is amplified by PCR using common primers in the flanking vector sequence (2), fluorescently labeled (3) and hybridized with an oligonucleotide microarray (4). The fluorescence pattern obtained (5) allows identifying the plasmid along 4 possibilities. Primer and oligonucleotide sequences are given in Appendix 1. The position of the oligonucleotide probes on the array is given in Appendix 4.

In order to give an example of fluorescence ratio experiments, two different plasmid preparations will be labelled with Cyanine 3 or Cyanine 5 fluorophores (Appendix 3), mixed and hybridized to a single microarray. The DNA labelling chemistry is explained in Appendix 2 and the probe immobilization chemistry in Appendix 5. Rationales in DNA hybridization are exposed in Appendix 6.
Step 1 : Plasmid DNA purification using the QIAprep spin miniprep kit

[1 h]

This protocol is designed for purification of about 1-5 µg plasmid DNA from 2 ml overnight cultures of XL-1 Blue E. coli in LB (Luria-Bertani) medium. All protocol steps should be carried out at room temperature. The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. The procedure consists of three basic steps:

- Preparation and clearing of a bacterial lysate
- Adsorption of DNA onto the QIAprep silica membrane
- Washing and elution of plasmid DNA at low ionic strength

This step can be skipped when plasmid DNA is already available.

1. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.
The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

2. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.
Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.

3. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.
The solution should become cloudy.

4. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.
A compact white pellet will form.

5. Apply the supernatants from step 4 to the QIAprep spin column by pipetting.

6. Centrifuge for 1 min at 13,000 rpm. Discard the flow-through.

7. Wash the QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 1 min.

8. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.
Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

9. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.
About 2-5 µg DNA are obtained, in 50 µL Elution Buffer.
Step 2 : Amplification of a DNA fragment of interest by PCR [2h30]

The fragment of interest carried by the plasmid is amplified using flanking primers contained in the pQE30 vector.

**Prepare premix for 3.5 reactions (20 min):**

<table>
<thead>
<tr>
<th>Volume (µL)</th>
<th>final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized H₂O</td>
<td>37.5</td>
</tr>
<tr>
<td>5 x GoTaq Polymerase buffer</td>
<td>13.5</td>
</tr>
<tr>
<td>dNTP mix 2mM</td>
<td>7</td>
</tr>
<tr>
<td>5’-primer (common 5’, 10µM)</td>
<td>3.5</td>
</tr>
<tr>
<td>3’-primer (common 5’, 10µM))</td>
<td>3.5</td>
</tr>
<tr>
<td>GoTaq Polymerase</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Prepare samples (10 min):**

<table>
<thead>
<tr>
<th>#1</th>
<th>#2</th>
<th>#3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premix</td>
<td>19 µL</td>
<td>19 µL</td>
</tr>
<tr>
<td>Plasmid DNA diluted 100 fold in H₂O</td>
<td>1 µL</td>
<td>1 µL</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Use 0.2 mL thin wall PCR tubes.

**Run PCR on thermocycler (1 h):**

Insert the tubes in the thermoblock, into the holes matching the tube size. Run the ‘TP’ program:

- 94°C 2 min  
  plasmid DNA denaturation
- 94°C, 30 sec  
  denaturation
- 50°C, 30 sec  
  annealing
- 72°C, 1 min  
  extension
- 20 cycles  
  extension of all generated fragments
- 68°C, 10 min  
  sample storage
- 4°C, indefinite time  
  sample storage

After PCR, store samples at 4°C and/or load on agarose gel for electrophoresis.

**Prepare 1% agarose gel in 0.5x TAE (10 min):**

In a 300 mL large opening Erlenmeyer flask, weigh 1 g agarose, add 95 mL deionized H₂O and heat up in a microwave oven (700 W, 1 min). When agarose is dissolved, wait until the solution has cooled down to about 60°C, add 5 mL of 10x TAE, mix slowly and pour the agarose solution into the gel tray within the casting stand. Insert the 13-teeth comb and let solidify for about 15 min at room temperature.

**Check DNA amplification by agarose gel electrophoresis (1 h):**

Remove the combs with both hands by gently pulling upwards. Transfer the gel tray in the electrophoresis Cell. Pour 0.5x TAE running buffer until the gel is fully submerged. About 350 mL of buffer are required.

For each sample, mix 5 µL of the PCR reaction with 1 µL loading dye and load in the gel.
Load also DNA molecular weight markers.

Connect the power cord of the Power Supply. Set the timer to ’30 min’ and the voltage to 100 V. Put the cell lid in place and press the output button to turn on the Power Supply. A blue LED will light on. The remaining time is indicated in min on the display.

**This part should be done only by trained experimentalists, since Ethidium Bromide and UV light are dangerous.**

When electrophoresis is finished, unplug the power cord, remove the lid and transfer the gel in a 0.5 μg/mL Ethidium Bromide solution for about 10 min. Wash the stained gel in water and observe under UV light illumination. Estimate the size and amount of the amplified DNA fragment by comparison with the molecular weight markers.

Mass Ruler DNA ladder (Fermentas Ref SM0403)
Step 3 : DNA labeling [2h30]

About 1 µg DNA (typically 20 µL PCR product) will be labelled in the procedure.

**Set the dry block heater to 95°C ! and the centrifuge to 4°C**

Preparing the labeling solution (optional, the solution may be ready) (5 min):

To prepare the ULS labeling reagent stock solutions, add 100 µL of 50% DMF (Component B) or 100 µL of DMSO (Component B), depending upon the kit, to the vial containing the ULS reagent (Component A). Vortex until all of the ULS labeling reagent has dissolved and no particulate matter remains. In order to completely dissolve the ULS reagents, vigorous vortexing, followed by pipetting up and down, may be required. DO NOT HEAT the solution. ULS reagent stock solutions may be stored at 4°C for up to six months.

DNA precipitation (1h30):

Precipitate 1 µg of DNA (30µL PCR product) by adding 1/10 volume (3 µL) of 3 M sodium acetate (pH 5.2) and two volumes (60 µL) of absolute ethanol, freeze at –70°C for 30 minutes and then centrifuge for 15 minutes at 12K rpm. Wash the pellet with 60 µL 70% ethanol and allow it to air dry. Resuspend the pellet in 20 µL of the labelling buffer (Component C).

DNA denaturation (10 min):

Denature the DNA at 95°C for 5 minutes and then snap cool on ice. Centrifuge the tube briefly to redeposit the sample to the bottom of the tube. Note: Denaturation is not absolutely required for labelling; however, it improves labelling efficiency by 20–40%.

**Set the dry block heater to 80 °C !**

DNA labeling reaction (20 min):

Add 5 µL of ULS labeling reagent stock solution to the tube containing the denatured sample DNA. If necessary, add labeling buffer (Component C) to bring the final volume to 25 µL.

Incubate the reaction at 80°C for 15 minutes. Stop the reaction by plunging the reaction tube into an ice bath. Centrifuge the tube briefly to redeposit the sample to the bottom of the tube.

Fluorescent DNA purification (15 min):

The DNA must now be purified by gel filtration from the excess ULS labeling reagent, using a BioRad Micro Bio-Spin® P-30 spin column. Silica-based separation technique should be avoided.

1 Invert the column sharply several times to resuspend the settled gel and remove any bubbles.
2 Snap off the tip and place column in a 2.0 ml microcentrifuge tube (included). Remove cap. Allow the excess packing buffer (10 mM Tris-HCl, pH 7.4 with 0.02% sodium azide) to drain by gravity to top of gel bed. If column does not begin to flow, push cap back into column and remove. Discard the drained buffer, then place the column back into the 2 ml tube.
3 Centrifuge for 2 min in a swinging bucket centrifuge at 1,000 x g (see Centrifugation Notes section) to remove the packing buffer. Discard the buffer.

4 Place the column in a clean 2.0 ml microcentrifuge tube. Carefully apply the sample directly to the center of the column. The 2 DNA samples that will be used for the same DNA chip should be purified together on the same column.

5 After loading samples, centrifuge the column for 4 min at 1,000 x g.

6 Following centrifugation, the purified sample is now in Tris buffer. The purified DNA solution is colored. Molecules smaller than the column’s exclusion limit will be retained in the void volume.
Step 4 : DNA hybridization with the oligonucleotide microarray [1h]

Thaw up in advance the 2x hybridization solution and prepare 100 μL of a 1x stock. Preheat the solution and the tubes at 50°C.

Pre-hybridization (30 min) : not absolutely required

This step is intended to block non-specific binding sites in the microarray.

Preincubate the oligonucleotide microarray glass slide at 43°C for 30 min with 50 μL or the 1x hybridization solution. Put the microarray in a wet chamber during the incubation to prevent drying.

In order to spread evenly the solution onto the slide, cut a piece of parafilm of about 1.5 cm x 4 cm. Put the solution dropwise on the slide. Gently lower the piece of parafilm onto the slide. Put one edge of the piece of parafilm down first, before lowering the rest of it. Do not press down on the piece of parafilm once it is in place.

After incubation, remove most of the hybridization solution by aspiration with a piece of paper.

Hybridization (overnight) :

Denaturation is necessary to separate the two strands of target DNA.

Denature the fluorescently labeled DNA solution for 3 min at 90°C. Mix 25 μL of the fluorescently labeled DNA solution with 25 μL of preheated 2x hybridization solution and spread immediately onto the microarray. Incubate overnight at 43°C.

Washing (30 min) :

Prepare the wash solutions of increasing stringency : 2x SSC, 0.1 % SDS and 0.2x SSC. Preheat the first solution to 50°C.

1st wash: 2x SSC, 0.1 % SDS
After the incubation, put the glass plate in a Petri dish and add the 2X SSC, 0.1 % SDS solution. The parafilm is released and floats on the solution. Wash during 2 min on the Rotamax shaker at 50 rpm.

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

Store in the dark.
**Step 5 : Fluorescence scanning [2h]**

Start the Amplireader and proceed as explained hereafter.
Scan the area of interest at a 15µm resolution and adjust the layout parameters to get fluorescence measurements. The image analysis software aims to detect a single spot of predefined size in each square of the grid. Check manually for the correctness of the spot identification. The quantification results are exported in an Excel spreadsheet where appropriate statistics can be performed.

1. **Set parameters**
   - Project : load “layout for Syn7_Syn8_Vti1_Vamp7.GWP”
   - Image : remove any image already present
   - Acquisition : select appropriate channels and define the acquisition time :
     - 1 sec : bright fluorescence
     - 10 sec : low fluorescence

2. **Acquisition**
   - Preview (optional)
   - Scan : 1sec -> 30 sec; 3 sec -> 100 sec
   - Menu pops up : rename ‘slide1’ (optional) and validate
   - Acquire next slide if you want to analyze all images together.

3. **Analysis**
   - Once the scan is completed, adjust MAX and MIN in the image menu to better see the spots.
   - Select the grid :
     - Settings of the grid (in ‘project’ menu) :
       - Layout :
       - Offset left 1151 top : 2871
       - Blocks :
         - Count columns : 2 rows : 7
         - Pitch vertical : 11000 horizontal : 9500
       - Pattern :
         - Count columns : 6 rows : 5
         - Pitch horizontal : 1500 vertical : 1500
     - Diameter of the spots : 300 µm
   - Run the image analysis program (“gears” icon). Check for the proper identification of the spots. Analyze the quantitative data in the spreadsheet.
Getting Started: first scan and first quantification

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

<table>
<thead>
<tr>
<th>Image control</th>
<th>Feature details</th>
<th>Layout editor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color mode</td>
<td>Cymine 3</td>
<td>Column</td>
</tr>
<tr>
<td></td>
<td>Cymine 5</td>
<td>Row</td>
</tr>
<tr>
<td>Ratio</td>
<td></td>
<td>Diameter</td>
</tr>
<tr>
<td>Pixel</td>
<td></td>
<td>Rotation</td>
</tr>
<tr>
<td>4% 20% 100%</td>
<td></td>
<td>0 - 10 degrees</td>
</tr>
</tbody>
</table>

#### How to adjust a layout?

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.

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.

#### Stretching the grid using the mouse

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

#### Viewing the Quantification Results

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.
Protein microarray analysis using SPRi

Surface plasmon resonance technology is described in Appendix 7. We are working with a SPRi-Lab+™ machine and gold surface prisms from Horiba Jobin Yvon (http://www.horiba.com). This equipment allows for label-free detection of biomolecules, real-time monitoring of kinetics curves, the determination of affinity-binding parameters and multiplexed imaging of different peptide spots grafted to the gold prism.

In these practicals we demonstrate the specificity of antigenic peptide-antibody interactions and quantify the kinetics of this interaction using SPRi.

Instrumental proceeding:

After having placed the prism correctly in the instrument, we begin by setting several parameters.

Determination of the resonance angle

In SPRi we work at a fixed angle (see Appendix 7) that has to be determined with respect to the resonance angle in our setup. The first setting to be made is therefore the determination of the resonance angle obtained with our prism.

Initialize the instrument and determine the folder where the experiment data will be saved. Next, flow the buffer in the chamber using the syringe pump at 50µL.min⁻¹. Then, launch the plasmon acquisition. After a few minutes, the acquisition is done and you are able to select and name the spots of interest. The software gives a curve giving the change of the prism surface reflectivity when the angle between the light beam and the surface increases. Check that this curve is not aberrant and select the angle which is adapted for the experiment. Once the resonance angle has been determined, the SPR signal is calibrated.

Calibration of the SPR signal on the prism

The surface of the prism cannot be considered as perfectly uniform and the SPR signal obtained in different parts of the prism can be altered because of variations in the material. Therefore a calibration has to be done that allows to average the SPR response obtained over the whole “visible” prism surface. A 0.25M NaCl 1% BSA solution is used for calibration. The injection of NaCl provokes a variation in refractive index and hence a variation in the reflectivity. The software measures reflectivity variation and uses it to calculate calibration coefficients that will be used to normalize spot responses in the kinetics step.

Let the buffer run until the signal is stabilized. During that time, inject the NaCl solution in the loop using a syringe (valve must be on the “Load” position). Then, change the valve to the “Inject” position. The signal increases very rapidly. Wait for the signal to be stabilized and stop the injection of the calibration solution. Wait for the signal to go back to the baseline and ask the software to calculate the calibration coefficients for each spot.

The experiment can now begin.
Injection of different antibody solutions

The following antibody solutions are available:
Monoclonal anti-c-myc Ab (Sigma Ref M4439)

They will be injected at different concentrations and flow rates:

Dilutions: 1/5000, 1/2000, 1/1000 in 25 mM Tris pH 7.4 125 mM NaCl 2 mM MgCl\(_2\) 1% BSA
Flow rates to test: 50 µL/min, 100 µL/min

Wait for the signal to be stabilized. During that time, fill the loop with the antibody solution and the valve position on “Load”. Then, change the valve position to “Inject”. Observe the signal change with the arrival of the antibody. After a few minutes (depending on the flow rate and the volume of antibody solution stored in the loop) stop the antibody injection and wash the surface with buffer.

Regenerate the surface with the regeneration solution (0.1M HCl, 1% BSA). Then the instrument is ready for the next antibody injection.

Analysis of association and dissociation kinetics

The determination of the association constant \(k_{\text{ass}}\) is graphically and needs at least 2 acquisitions with different antibody concentrations. Plotting the initial binding rate against the corresponding antibody concentration gives a linear relationship. The slope of this straight line is equal to:
\[
X_{\text{slope}} = S_{\text{max}} \times k_{\text{ass}}
\]

where \(S_{\text{max}}\) is the maximum signal at the highest antibody concentration.

The dissociation constant \(k_{\text{diss}}\) can be calculated using the equation:
\[
S_t = (S_0 - B)[\exp(-k_{\text{diss}}t)] + B.
\]

where \(S_t\) is the signal during dissociation at point t (in second), \(S_0\) the initial signal before dissociation and B is the baseline signal after the regeneration of the surface.

Having determined \(k_{\text{ass}}\) and \(k_{\text{diss}}\), a calculation of \(K_D\) is possible using the equation
\[
K_D = k_{\text{diss}}/k_{\text{ass}}.
\]

Map and multiple cloning site of the pQE30 vector. Sequences of interest are inserted using the BamHI and KpnI restriction sites.

Sequences of pQE30:Syn7, pQE30:Syn8, pQE30:Vti1, pQE30:Vamp7 plasmids. The common 5' and 3' primers and the specific oligonucleotide probes are indicated. The vector sequence is in green. The common primers are used both to amplify the sequence of interest by PCR and for hybridization. The common primers used in PCR lack the 5’ NH2 linker.

pQE30:Syn7:

61 ...ATTGTGAGCG GATAACAAAT TTCACTACAA TTCTATTAAAG AGGGAAATTT
AATCTATGAGGATGCCATACCACATCCACATCCGATCCTGACAAAAATTTCTGACTATC
ATCAACTACATGAAATATTTCAACAAAAATGCTGACATAATTTAACCATATTTAGTT
CAACTATGGGATACCCAATCAAATCGTAAATGTTAGAAAGAAATCGTAAATGTTAGTGA
TTCAACAACATTTTTATTTCACTACGAAATCTGGCAGAAAGTTAAAAACCTTACATCTTTAGCAA
GTCGTTCACGTAGTTAAAATATTTTATATATATATAAATAGTTAAGAATTTATTATAT
TGTTTACAAATATATTTGACCAAAGTTGAAAATATGAGATTTGGAAGAAACGCAAACAA
ATATTTACAAATATATACACCAAAAAATATTTGCAAAATACACAAACAGCTTAAATTTAC
AAGAGACCGATACCAAAAAATATTTGAGAAGAATGCATACACAAACAGCTTAAATTTAC
AAGAGACCGATACCAAAAAATATTTGAGAAGAATGCATACACAAACAGCTTAAATTTAC
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AAGAGACCGATACCAAAAAATATTTGAGAAGAATGCATACACAAACAGCTTAAATTTAC
pQE30:Syn8

61 ...ATTGTGACG CATAAACATT TACACAGAA TTCATTTAAC AGGAGAAAAT
AATCTATGAGAGAGATCCATACCATCACCATACCCAGGATCATGAGATAATAATATATAAAAA
ATTGTAATTAATAGTAGTTATATGTTTATATGACTATATATATTTTTAT
AAATGAAATTTTATGATATCAGAACTCTGTTGAC

pQE30:Vti1

61 ...ATTGTGACG CATAAACATT TACACAGAA TTCATTTAAC AGGAGAAAAT
AATCTATGAGAGAGATCCATACCATCACCATACCCAGGATCATGAGATAATAATATATAAAAA
ATTGTAATTAATAGTAGTTATATGTTTATATGACTATATATATTTTTAT
AAATGAAATTTTATGATATCAGAACTCTGTTGAC

pQE30:Vamp7

61 ...ATTGTGACG CATAAACATT TACACAGAA TTCATTTAAC AGGAGAAAAT
AATCTATGAGAGAGATCCATACCATCACCATACCCAGGATCATGAGATAATAATATATAAAAA
ATTGTAATTAATAGTAGTTATATGTTTATATGACTATATATATTTTTAT
AAATGAAATTTTATGATATCAGAACTCTGTTGAC
The Universal Linkage System (ULS™) technology (Kreatech) allows DNA, RNA and protein labeling for FISH, array CGH, miRNA microarrays, gene expression microarrays, protein arrays and others. ULS labeling is based on the stable binding properties of platinum(II) to nucleic acids and proteins [1-3]. The ULS molecule consists of a monofunctional platinum complex coupled to a detectable molecule of choice. The platinum atom forms a coordinative bond, firmly coupling the detectable molecule to the biomolecule of choice: DNA, RNA or protein. ULS labels DNA and RNA by binding to N-7 of guanine. In proteins, ULS binds to sulfur-containing side chains of methionine, and cysteine, and to a nitrogen atom in histidine (see figures below).

Schematic Overview of the ULS Technology

References


APPENDIX 3 : FLUORESCENT DETECTION OF CY3- OR CY5-LABELED DNA

Cyanine 3 and Cyanine 5 fluorescent chromophores

The R-group is used to attach these chromophores to molecules of interest, e.g. target DNA.
Fluorescence spectra of CY3 and CY5 dyes

Cy3 is excited maximally at 550 nm 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 near-infra red part of the spectrum; quantum yield is 0.28. FW=792.

Fluorescence spectra of AlexaFluor546 and AlexaFluor647

The ULS Nucleic acid labeling kit uses AlexaFluor546 and AlexaFluor647 fluorophores, whose fluorescent properties are close to those of Cyanine 3 and Cyanine 5.

AlexaFluor546 is excited maximally at 556 nm and emits maximally at 573 nm, in the red part of the spectrum; extinction coefficient is 104000.

AlexaFluor647 is excited maximally at 650 nm and emits maximally at 665 nm, in the near-infra red part of the spectrum; extinction coefficient is 239000.
ampliREADER™
Compact CCD-based Microarray Reader

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 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 footprint: only 26 x 35 cm (10x14 inches)

Key Features
- Original wide-field optical architecture
- Fast acquisition time
- Dye compatibility: Cy®3 & Cy5 and analogous
- High quantum efficiency cooled CCD
- Integrated barcode reader
- OEM versions and other wavelengths on demand
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

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

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<tr>
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<tr>
<td></td>
<td>• Cy3 and Cy5</td>
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<tr>
<td></td>
<td>• Alexa®546 and Alexa 647</td>
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<tr>
<td></td>
<td>• DY®-548 and DY-648</td>
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Ordering information

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<td>AmpliReader 4800, 1-channel Green (535nm), Array4D™ included</td>
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<td>4AR4600-D640</td>
<td>AmpliReader 4800, 1-channel Red (640nm), Array4D™ included</td>
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</table>
APPENDIX 4: COVALENT BINDING OF DNA PROBES TO FUNCTIONALIZED GLASS SUBSTRATES

Oligonucleotide probes

As explained in Appendix 1, the sequence of the oligonucleotide used for the identification of the plasmid DNA are:

<table>
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<tr>
<th>Oligo name</th>
<th>Oligo sequence</th>
<th>Length</th>
<th>%GC</th>
<th>MW</th>
<th>calc Tm</th>
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<tr>
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<td>TCGCATCACCATCACC</td>
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<tr>
<td>pQE30:Syn7</td>
<td>ACCATTACCAGTTGCACC</td>
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<td>5405</td>
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<td>pQE30:Vti1</td>
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<td>ACAGCTACATACGACCCA</td>
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<td>50</td>
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<td>Common 3'</td>
<td>GAGCTTGGACTCCTGTTG</td>
<td>18</td>
<td>50</td>
<td>5523</td>
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</tr>
<tr>
<td>Std</td>
<td>GATAAACCCACTCTA</td>
<td>15</td>
<td>40</td>
<td>4506</td>
<td>39</td>
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<td>1M</td>
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<td>GATAAAGACACTCTA</td>
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Oligonucleotides were chemically synthesized and covalently modified at the 5’-terminus by an amine (Genosys, Sigma). The DNA concentration in the stock solution is about 200 µM. Each probe was diluted to 10 µM in 0.3 M sodium phosphate solution (50 µL final volume) and transferred in the 384-well sample plate of the robot, according to the following scheme.

As an alternative in the case of poor DNA labelling, four probes are also spotted on the microarray:

- “standard” sequence : “Std” NH₂-TTTTTTGATAAACCCACTCTA
- standard sequence with one mismatch: “1M” NH₂-TTTTTTGATAAAGCCACTCTA
- standard sequence with two mismatches: “2M” NH₂-TTTTTTGATAAAGACACTCTA
- “unrelated” sequence “X” NH₂-TTTTTTTTTCCAAGAAAGGACCG

Under this possible event, the microarray will be hybridized with the following pre-labelled DNA oligonucleotide:

- “target DNA” Cy3-CATAGAGTGGGTTTATCCA

The melting temperature of the oligonucleotide probes used in the microarray were calculated using the Biomath calculator at the Promega web site:

http://www.promega.com/biomath/calc11.htm
Spotting layout

Probe arrangement in the DNA tray

<table>
<thead>
<tr>
<th>Position</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
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<td>1M</td>
<td>Syn</td>
<td>Vti1</td>
<td>Com 5'</td>
</tr>
<tr>
<td>2M</td>
<td>X</td>
<td>Syn</td>
<td>Com 3'</td>
</tr>
<tr>
<td>Std</td>
<td>Syn</td>
<td>Vamp</td>
<td>Com 3'</td>
</tr>
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</table>

Basic pattern layout (pitch = 1.5 mm, 3 x 2)

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<th>2</th>
<th>3</th>
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</thead>
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<td>Com 5'</td>
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</tr>
<tr>
<td>3</td>
<td>Syn</td>
<td>Vamp</td>
<td>Com 3'</td>
</tr>
</tbody>
</table>

Slide layout (spacing = 1 mm)

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<th>3</th>
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<td>Vti1</td>
<td>Com 5'</td>
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<tr>
<td>2</td>
<td>X</td>
<td>Syn</td>
<td>Com 3'</td>
</tr>
<tr>
<td>3</td>
<td>Syn</td>
<td>Vamp</td>
<td>Com 3'</td>
</tr>
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Oligonucleotide pattern on the microarray

<table>
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</tr>
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<tbody>
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<td></td>
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</tbody>
</table>

4.5 mm
Glass substrates

DNA microarrays are commonly prepared on glass surfaces, typically aminosilane-coated microscope slides. Genewave (http://www.genewave.com/) sells ‘Amplislide’, glass plates that are coated with a multi-layer thin film to enhance the fluorescence signal, resulting in a higher sensitivity (better signal/noise ratio).

More information is available at Genewave web site:
Silane activation (see chemical reaction scheme in appendix 5)

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 wafer and amino-modified DNA oligomers deposited by the robot pins.

- 1\textsuperscript{st} treatment:

  This pre-treatment is required when the glass plates have remained several days in contact with ambient air after silanization. \( \text{CO}_2 \) contained in air reacts with terminal \( \text{NH}_2 \) and forms \( \text{NH}_2\text{CO}_3 \) which prevents efficient coupling. This reaction is reversed by KOH treatment.

  - Immerse the silicon plates in a 0.1 M KOH aqueous solution during 15 min
  - Rinse thoroughly with deionized water and dry with compressed air

- 2\textsuperscript{nd} treatment

  - Immerse the silicon plates in a 10\% glutaraldehyde (Sigma G-7651) aqueous solution and gently agitate during 1h30 at room temperature (rotamax).

**Be careful not to breath toxic glutaraldehyde fumes. Wear gloves and labcoat. Dispose glutaraldehyde waste properly.**

  - Rinse with deionized water and dry with compressed air.

Spotting operation

The probes are spotted in on the glass slides using four solid pins (0.1 mm). The spot size is about 300 µm.

The parameters of the spotting robot are: the type of sample (glass plates), the number of probes (12), the number of pins used (4), their type, the spotting velocity, the basic motif and the number of repeats, the washing procedures. The glass plates are held on the robot moving plate by vacuum suction. When all parameters have been defined, the spotting procedure is launched. The microarrays are then incubated overnight at room temperature in saturating water.

The next morning, a stabilization treatment is performed by the CIME technical staff.

  - \( \text{NaBH}_4 \) reduction of pending aldehyde bonds (\( \text{CHO} \rightarrow \text{CH}_2\text{-OH} \)) and N=C stabilization.
  - Stringent washing with an anionic detergent (0.2\% SDS) to remove the probes not covalently bound to the substrate.

The DNA microarrays 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.
APPENDIX 5: CHEMICAL COUPLING OF DNA PROBES TO A GLASS SURFACE

HYDROXYLATION

SILANIZATION

γ-APTES

Curing

Atmospheric CO₂ INACTIVATION

RE-ACTIVATION

HOC(CH₂)₃COH
OLIGONUCLEOTIDE GRAFTING

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

STABILIZATION TREATMENT

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

HYBRIDIZATION AND DETECTION

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

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\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

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\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]

\[ \text{Si} \] \[ O=C - H \] \[ (\text{CH}_2)_3 \] \[ O \] \[ \text{N} \] \[ (\text{CH}_2)_3 \] \[ O=\text{Si} \] \[ O-Si-O \] \[ O \] \[ \text{N} \]
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.

In this lab work, the direct detection method (left panel) will be used.

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.

\[
\text{amine} \quad \text{aldehyde} \\
\text{H}_2\text{O} \quad \text{R}-\text{CH}_2\text{-NH}_2 + \text{R’-CH}_2\text{-C}=\text{O} \xrightarrow{\text{H}} \text{R}-\text{CH}_2\text{-NH}^-\text{C}^-\text{CH}_2\text{-R’} \\
\text{Instable imine} \quad \text{Schiff base} \\
\text{NaBH}_4 \quad \text{R}-\text{CH}_2\text{-N=CH}_2 \xrightarrow{\text{H}_2\text{O}} \text{R}-\text{CH}_2\text{-NH}^-\text{C}^-\text{CH}_2\text{-R’} \\
\text{Stable amine}
\]

\[\text{γ-aminopropyltriethoxysilane (APTES)}\]

**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$_n$Si(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.
The complementary 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 in 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, 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 a nucleic acid complexes depend on the G,C content of the sequence involved in their binding. It should be noted that the association between two nucleic acid molecules does not require perfect complementarities and the presence of mismatches simply decreasing the total binding energy.

The presence of similar gene products in genomes, 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).

This curve is called a melting curve and the melting temperature $T_m$ is the temperature at which half of the targets are bounded to probes. The melting temperature depends on the G,C 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://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 $T_m$ by 1.5 to 10 °C, for 100 to 20 bp oligonucleotides respectively. For larger DNA molecules, a commonly used formula is that of Bolton and MacCarthy (1962):

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

where $N$ is the nucleotide number.

This equation only works for Na+ concentrations of 1 M or less. It has been shown experimentally to hold 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 group on the strand backbone get charged, resulting in strand separation. Note that alkaline denaturation is not used for RNA molecules, since this also favors hydrolysis of the phosphodiester bond along the strand.

**Ion concentration (ionic strength):** at neutral pH, negative charges are present on the phosphate groups along the 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 \( \pi \)-electrons of the pyrimidine and pyrene 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 denaturating 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 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. Longer washing times will increase the specificity, but as the expense of the magnitude of the fluorescent signal recorded. This explained 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.
Surface Plasmon Resonance in imaging mode (SPRi)

Surface plasmons are defined as the collective oscillations of electrons which exist at the interface of two different materials, like a metal (usually gold) and a dielectric (usually an aqueous solution).

Any SPR set-up (see below left) can be broadly divided into three major parts:
1. The optical part, which comprises the light source and an optical detector (in the imaging mode a camera).
2. The flow cell which is the section for passing solutions over the gold surface; it has an inlet to inject and an outlet to collect the sample.
3. The gold-dielectric interface provided by a prism put into contact with the flow cell.

The phenomenon of surface plasmon resonance occurs when photons of incoming light excite the surface plasmons of the gold layer and their resonance leads to energy and momentum transfer from photons to plasmons creating an evanescent wave at the gold-dielectric interface. The resonance between the incoming photons and the plasmons occurs only at a specific resonance angle.

At this angle a minimum is observed in the reflectivity vs. angle curve (see top right), and this characteristic curve is called the Plasmon curve. The minimum signifies that no reflected light is detected as all the energy of the incoming light has been transferred to the surface plasmons. This phenomenon is very sensitive to changes in the optical properties of the dielectric (refractive index changes, mass adsorption). The variation of the reflectivity with time is plotted in a sensogram (bottom right) that allows assessing the kinetics and quantity of mass adsorption on the gold surface. In the SPRi system, the SPR signal is recorded at a fixed angle chosen by the operator and the registration of a sensogram is
coupled to a real-time image of the surface of the gold prism.

For protein interaction studies, one of the two interacting partners is grafted covalently at the gold surface while the other is presented in solution via the flow cell. Typically an antigenic peptide can be grafted and its specific interaction with an antibody in solution can be measured. Adsorption kinetics are obtained when the flow cell is flushed with the antibody dissolved in buffer. A steady state will be reached when the amounts of antibody binding to and unbinding from its peptide antigen are equal. Desorption kinetics are measured when the flow cell is flushed with buffer alone (see below). The variation in SPR signal is proportional to the mass present at the sensor surface. A gold prism surface can be regenerated for consecutive experiments using an accurate buffer.

\[
\begin{align*}
\text{k}_{\text{association}} &= \text{k}_{\text{on}} [B] \\
\text{k}_{\text{dissociation}} &= \text{k}_{\text{off}} \\
\text{K}_{\text{affinity}} &= \text{k}_{\text{off}} / \text{k}_{\text{on}}
\end{align*}
\]

The main advantages of SPR to study protein interactions are, that there is no need for labelling nor purification (whole sera could be used) and the sensitivity is very high.
APPENDIX 8 : Prism preparation

Preparation of the gold coated prism and grafting of antigenic peptides

Gold coated prisms (SPRi Biochips) are obtained from Horiba. The prism is first cleaned using liquid soap, ethanol and deionised water and is then dried by centrifugation (4000 rpm for 3 minutes). An oxygen plasma treatment for 5 minutes insures that all organic contaminants are eliminated. Immediately after the prism is functionalized with SH- aminesilane, and glutaraldehyde (give details of substances, buffers, incubation times etc).

The antigenic peptides used are the following (antigenic sequence in bold):

Peptide K-linker-c-myc :
NH2 - KGGSGGSGYGSGSEQKLI SEEDL - COOH

They are diluted in PBS buffer. The glutaraldehyde chemistry detailed in appendix 5 is used to create a covalent link between the prism gold surface coated with aminesilane and the N-terminal amines of the peptides.

The peptides are spotted onto the prepared prism using the Microgrid II robot (file: prismeSPRmycHis18.08.11). They are aligned as follows in a 9x9 array with 0.5mm spacing:

<table>
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<tr>
<th></th>
<th>0</th>
<th>His 32 µM 0</th>
<th>0</th>
<th>His 16 µM 0</th>
<th>0</th>
<th>His 8 µM 0</th>
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<th>His 4 µM 0</th>
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<tr>
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<td>His 32 µM 0</td>
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<td>His 16 µM 0</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>0</td>
<td>Myc 32 µM 0</td>
<td>0</td>
<td>Myc 16 µM 0</td>
<td>0</td>
<td>Myc 8 µM 0</td>
<td>0</td>
<td>Myc 4 µM 0</td>
<td></td>
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<tr>
<td>0</td>
<td>0</td>
<td>Myc 32 µM 0</td>
<td>0</td>
<td>Myc 16 µM 0</td>
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<td>Myc 4 µM 0</td>
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</tbody>
</table>

Change this table as we will only use c-myc
Peptide drops are left overnight in a wet atmosphere (PBS) and a NaBH4 reduction is done the next morning on the gold surface. The peptide-spotted prism can be stably stored at 4°C.
**COMPOSITION OF STANDARD HYBRIDIZATION AND WASHING SOLUTIONS:**

Control of the pH and ionic conditions is usually achieved by using 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 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 (Sodium Salt Phosphate EDTA)

- **For 1 Liter**
  - 175.3 g sodium chloride
  - 27.6 g NaH2PO4-H2O
  - 7.4 g EGTA
  - adjust 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 denatured 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 solution are made by diluting an unlabeled DNA solution in 5x to 6x SSC or SSPE and 1x to 5 x Denhardt’s solutions. Here is shown the composition of the hybridization solution used in the lab work:

**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
Material needed and ordering references

Materials or consumables

<table>
<thead>
<tr>
<th>Material</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>QIAPrep Spin Miniprep kit (50 react)</td>
<td>Qiagen 27104</td>
</tr>
<tr>
<td>DNA Tap Polymerase and buffer (GoTaq 100 µL, 5U/µL)</td>
<td>Promega M3171</td>
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<tr>
<td>dNTP mix</td>
<td></td>
</tr>
<tr>
<td>5’ primer</td>
<td>Sigma Genosys</td>
</tr>
<tr>
<td>3’ primer</td>
<td></td>
</tr>
<tr>
<td>Agarose, NEEO</td>
<td>Roth 2767.2</td>
</tr>
<tr>
<td>10 x TAE</td>
<td></td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td></td>
</tr>
<tr>
<td>100% Ethanol</td>
<td></td>
</tr>
<tr>
<td>70% Ethanol</td>
<td></td>
</tr>
<tr>
<td>3 M Na Acetate, pH 5.2</td>
<td></td>
</tr>
<tr>
<td>Ulysis nucleic acid labeling kits</td>
<td>436€ for 20 labelings</td>
</tr>
<tr>
<td>Alexa Fluor 546 (equivalent to Cy3)</td>
<td>Invitrogen U21652</td>
</tr>
<tr>
<td>Alexa Fluor 647 (equivalent to Cy5)</td>
<td>Invitrogen U21660</td>
</tr>
<tr>
<td>Amplislides 570/670 CCD</td>
<td>Genewave 180 A INB-05</td>
</tr>
<tr>
<td>oligonucleotide probes (NH2-linker)</td>
<td>Sigma Genosys</td>
</tr>
<tr>
<td>50% glutaraldehyde solution</td>
<td>Roth 4995.1</td>
</tr>
<tr>
<td>0.3 M Na phosphate solution</td>
<td></td>
</tr>
<tr>
<td>SSPE (or SSC) hybridization solution (2x)</td>
<td>Sigma H2266</td>
</tr>
<tr>
<td>Pipetmans</td>
<td></td>
</tr>
<tr>
<td>Microcentrifuge</td>
<td></td>
</tr>
<tr>
<td>Mupid DNA electrophoresis gel system</td>
<td></td>
</tr>
<tr>
<td>PCR thermocycler + 0.2 mL tubes</td>
<td></td>
</tr>
<tr>
<td>Dry block heater</td>
<td></td>
</tr>
</tbody>
</table>

Recipes

10x TAE quantity for 500 mL final concentration
24.2 g Tris base 0.4 M
6 mL acetic acid 0.2M
10 mL 0.5 EDTA-Na 0.01 M

0.3 M Na-Phosphate
**MicroGrid II spotting robot operation**

Switch on the power supply underneath the robot  
Unlock the security latches (red buttons on both sides of the robot)  
Switch on the multiple plug (screen and light)  
Switch on the computer. Username and password : enspg  
Launch TAS application suite (MGII compact).  
Look at the top line menu and check for the water lever.  
Open 'sept 2009' then select the ‘glass plate new DNA lab’ file  
Start the program with the ‘Go’ button

**Here are screenshots of the different parameter menus**

Adjustable parameter : spots before refill

The **number of probes** is defined here, 12, corresponding to 3 positions of the tool in the DNA tray.
This editor is used to define the pattern of spots in a block. These numbers point to the tool position in the DNA probe tray.

This editor is used to define the pattern of blocks in the slide.

The ‘soft touch’ menu is used to define how the pins get into contact with the glass surface.