

DNA & Protein Microarrays

Prof. Steven S. Sallierman, <http://sallierman.umn.edu/>



Overview

- DNA microarrays
 - Looking for gene mutations with DNA
 - Studying gene expression with mRNA
 - Fabrication
 - Polymerase chain reaction (PCR)
 - Reverse Transcription PCR
- Proteomics
 - From amino acids to proteins.
 - Traditional protein experimentation
 - Protein microarrays types.
 - Fabrication
 - Factors affecting performance.
- Appendix
 - Eukaryotic gene regulation & post-translation modification (PTM).
 - Protein studies.
 - Examples of DNA lab-on-a-chip devices.

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DNA to Protein



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<https://youtu.be/g7uCsKU0hA>

DNA Microarray



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Gene Expression with mRNA and cDNA...

DNA Microarray

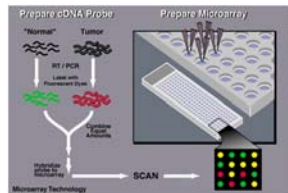


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<https://youtu.be/NgR6a0X08>

Looking for Gene Mutations...

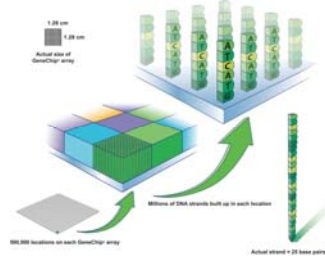
1. Start with a sample of DNA from blood, and a control sample without the specific gene mutation.
2. Denature the DNA into two complimentary single-stranded molecules.
3. Cut the strands into smaller fragments and label the patient's sample with green dye and the control with red dye.
4. A DNA chip with synthetic probes for both normal and mutated genes is utilized.
5. Both samples are placed into the chip and allow to hybridize (bind) to the synthetic DNA probes on the chip.



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Affymetrix GeneChip and Probes...



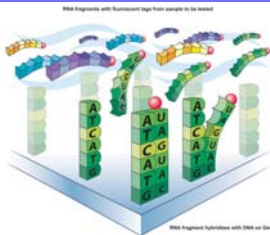
The GeneChip array measures 1.25 cm² and is subdivided into 11 μm squares or features each holding an array of just one unique type of probe.

An array of probes makes a feature, and an array of features makes the total chip).

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Images courtesy of Affymetrix

Hybridization...

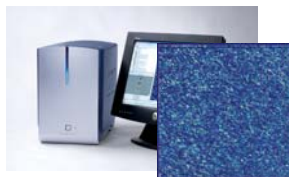
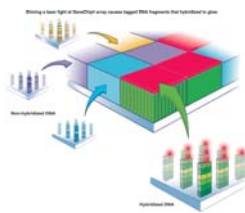


The sample is washed over the array for 14-16 hours to allow hybridization to occur. This process allows the chemical bonding of the DNA probes with the matching RNA fragment.

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Images courtesy of Affymetrix

Fluorescence Measurement...

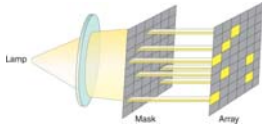


The amount of fluorescence in a given feature correlates with the amount of RNA that was present in the original sample. If there is no fluorescence over a feature, then there is no matching RNA present (above). Lighter areas represent increased expression (right).

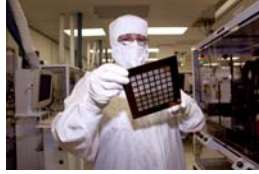
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Images courtesy of Affymetrix

Fabrication of the DNA Microarray



GeneChip probe arrays are manufactured through a combination of photolithography and combinatorial chemistry.

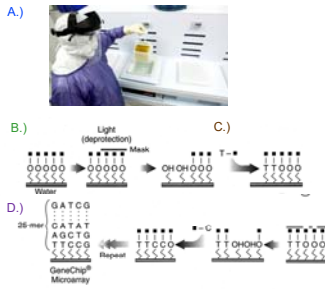


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Images courtesy of Affymetrix

Silanation of Silicon Surface & Probe Synthesis...

- A. Initially the quartz is washed to ensure uniform hydroxylation across its surface.
- B. Linker molecules, attached to the silane matrix will participate in nucleotide coupling after exposure (via mask) to UV light.
- C. A solution containing a single type of nucleotide with a removable protection group is flushed over the wafer's surface.
- D. Probe synthesis occurs in parallel, resulting in the addition of an A, C, T, or G nucleotide to multiple growing chains simultaneously.



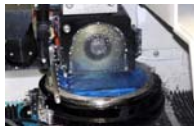
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Inspection, Dicing and Packaging...

Wafer Inspection



Dicing



Packaging



Final Inspection



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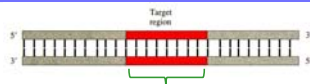
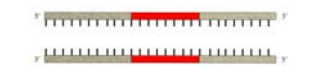
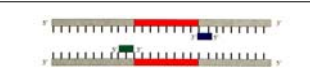
Images courtesy of Affymetrix

Polymerase Chain Reaction (PCR)

- An in vitro method of replicating small DNA sequences into millions of copies over a short period of time.
 - Nanomolar quantities of DNA can be replicated within a few hours.
 - PCR may be used for genetic testing in disease diagnosis, monitoring response to treatment, and tissue typing.
- A typical PCR requires:
 - Two oligonucleotide **primers**;
 - A thermally stable **DNA polymerase**;
 - Supply of **free nucleotides**; and
 - A small amount of DNA sample that contains the **sequence of interest**.
- The DNA fragment of interest must be known, so that short DNA primer fragments can be synthesized in advance, and are complimentary to the 3' end of each sample stand.

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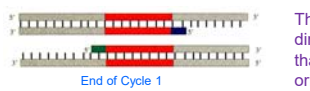
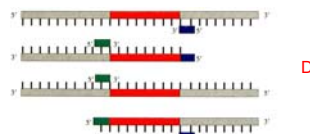
Denaturing and Annealing Steps...

- a)  Desired sequence or target region to amplify.
- b)  Denaturing by heating 95 C
- c)  Annealing by cooling to 60 C, and binding of primers.

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Carey, FA, Advanced Organic Chemistry 5th Ed., 2002

Synthesis and Denaturing...

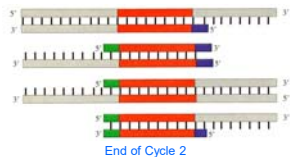
- d)  End of Cycle 1 The primer is extended in its 3' direction as it adds nucleotides that are complementary to the original DNA strand.
- e)  Denature again and prime.

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Carey, FA, Advanced Organic Chemistry 5th Ed., 2002

Elongation and Sequence Amplification...

f)



End of Cycle 2

Elongation of the primed polynucleotide fragments completes the second cycle and gives 4 DNAs.

g)



Among the eight DNAs formed in the third cycle are two having the structure shown. This structure increases disproportionately in the succeeding cycles.

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Carey, FA, *Advanced Organic Chemistry 5th Ed.*, 2002

DNA Amplification Results...

Cycle Number	Total Number of DNAs	Number of DNAs Containing Only the Target Region
0	1	0
1	2	0
2	4	0
3	8	2
4	16	8
5	32	22
10	1,024	1,004
20	1,048,566	1,048,526
30	1,073,741,824	1,073,741,746

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Carey, FA, *Advanced Organic Chemistry 5th Ed.*, 2002

Reverse Transcription PCR

- mRNA rather than DNA is used as the starting template.
- Useful for RNA pathogens like HIV and Hepatitis C, gene expression, and differentiating viable from nonviable cells.
- Step 1
 - The enzyme *reverse transcriptase* uses the mRNA template to produce a complementary *single-stranded DNA* strand called *cDNA* in a process known as *reverse transcription*.
- Step 2
 - *DNA polymerase* is used to convert the single-stranded cDNA into *double-stranded DNA*. These DNA molecules can now be used as templates for a PCR reaction.

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Matt Carter, Jennifer Shieh, in *Guide to Research Techniques in Neuroscience (Second Edition)*, 2015

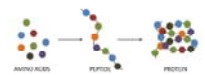
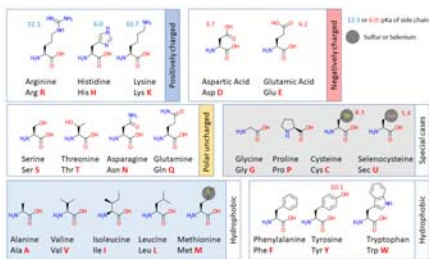
Proteomics

- The study of all proteins, including their:
 - Relative abundance
 - Distribution
 - Post-translational modifications
 - Functions
 - Interactions with other macromolecules
- In a given *cell or organism* within a *given environment* and at a *specific stage in the cell cycle*.

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Pardanani, A. et al. Mayo Clinic Proceedings 77(11), 2002

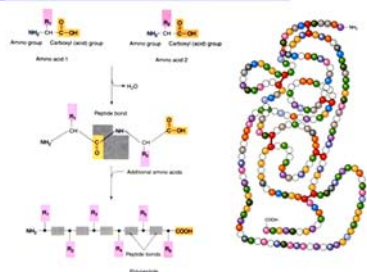
Amino Acids...



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Proteins...

- Proteins are made from the linkage of *amino acids* by peptide bonds to form a polypeptide.
- If there are less than 50 amino acids it is called a peptide, and if greater than 50 amino acids it is called a protein.
- The position of each type of amino acid in a polypeptide chain and the total number of amino acids in the chain distinguish one polypeptide from another.



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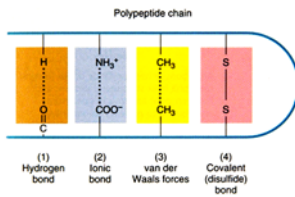
Vander, A.J. et al., Human Physiology: The Mechanism of Body Function, 8th Ed., 2001

Proteome...

- The entire collection of proteins, estimated to be more than 100,000.
- More proteins comprise a proteome than genes a genome.
 - Alternative *gene splicing* of mRNA,
 - *Posttranslational modification* (PTM).
- There is neither a one to one correlation of gene to protein, nor mRNA levels to proteins levels.
- PTM and signal transduction play a major role in cell transformation, such as tumor cells.
 - Specific genes are turned on or off at the onset of initiation, development, and progression of diseases such as cancer.
 - Signal transduction, via growth factors, proteins, and peptides, plays a major role in cell transformation (e.g. carcinogenesis).

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Bonding and Tertiary Structure...



Myoglobin

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Vander, A.J. et al., Human Physiology: The Mechanism of Body Function, 8th Ed., 2001

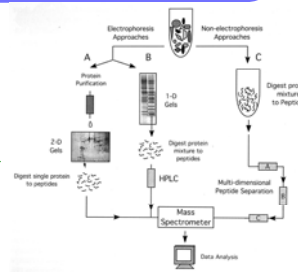
Post-translational Modification...

- Post-translational modification (PTM):
 - Phosphorylation, glycosylation, acetylation, ubiquitination, methylation etc.
 - PTM of proteins, not detected through RNA analysis, may occur at different stages of tumor development indicative of early or late events of transformation.
 - High throughput techniques may useful for screening and surveillance.

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Traditional Protein Experimentation...

- A. Protein purification may be performed by affinity chromatography, ion exchange, or subcellular fractionation. Then, 2-dimensional gel electrophoresis allows isolation of single proteins which may be digested for MS.
- B. Use of 1-dimensional gel allows partial electrophoretic separation, requiring further separation such as high-performance liquid chromatography (HPLC).
- C. The entire mixture is digested to peptides and the peptide mixture is resolved by multi-dimensional chromatography.



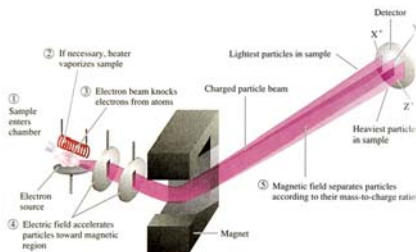
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Graves, PR and TAJ Haystead, Handbook of Proteomic Methods (2003)

Separation is Based on Mass-to-Charge Ratio...



Mass Spectrometer



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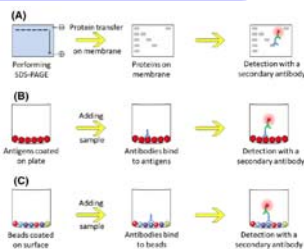
Carey, FA, Advanced Organic Chemistry 5th Ed., 2002

Clinically Useful Identification of Proteins...

Western Blot - separation of proteins according to their length and isoelectric point using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) followed by transfer to a membrane.

ELISA - wet samples incubated with specific antibodies in a microtiter plate. Detection is in wells.

Bead-based Method - e.g. Luminex® is a bead-based method that can analyze multiple samples in one experiment by utilizing different bead types.



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Abel, L. et al. Autoimmune profiling with protein microarrays in clinic applications. Biochimica et Biophysica Acta 1844 (2014) 977-987

Areas of Interest...

- 1) **Abundance proteomics:**
 - Relative abundance of specific proteins in a given tissue under different conditions of health and disease.
- 2) **Cell-mapping:**
 - Intracellular signaling pathways and regulatory networks mediated through protein-protein interactions.
- 3) **Structural proteomics:**
 - Study of active sites and functional domains of proteins to better understand processes such as enzyme catalysis, protein stability and interaction with multi-molecular complexes.

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Protein Microarray Types

- **Forward-phase microarrays (or Analytical):**
 - Proteins and peptides are immobilized for capturing antibodies.
 - Antibodies, sugars or aptamers are immobilized and labeled proteins are captured.
 - Sandwich mode – a labeled secondary antibody is used for detection.
- **Reverse phase microarrays:**
 - Complex samples such as serum, plasma, or even tissues are immobilized in an array format and probed with antibodies to determine the differential amount of protein molecules in the screened samples.

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Forward Phase Protein Microarray (or Analytical)...



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<https://youtu.be/9TYgNWyENRE>

Protein Microarray Uses...

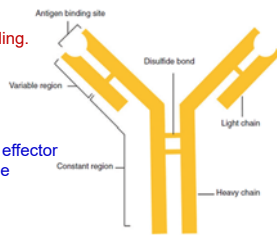
- Protein expression profiling.
- Studying the following:
 - Posttranslational modifications.
 - Protein-protein binding.
 - Drug interaction.
 - Protein folding.
 - Substrate specificity.
 - Enzymatic activity.
 - Interaction between proteins and nucleic acids.

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Antibodies

Responsible for Ag binding.

Interacts the with various effector molecules, which mediate the immune response.

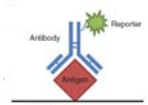


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Adopted from Spósito A.J., Kurdekar A., Zhao J.Q., Hewlett I. Application of nanotechnology in biosensors for enhancing pathogen detection. *Wiley Interdisciplinary Reviews-Nanomedicine and Nanobiotechnology*. 2018;10(5).

Antigen Identification...

Direct immunoassay



Dye, nanoparticle, enzyme etc.

Binding to the exposed *epitope* of the immobilized antigen.

Indirect immunoassay



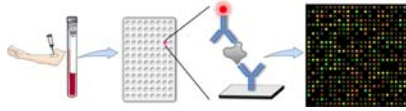
Antigen affixed to substrate.

Multiple secondary antibodies bind to the original antibody, amplifying the signal.

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Adopted from Spósito A.J., Kurdekar A., Zhao J.Q., Hewlett I. Application of nanotechnology in biosensors for enhancing pathogen detection. *Wiley Interdisciplinary Reviews-Nanomedicine and Nanobiotechnology*. 2018;10(5).

Microarray Analysis Concept...

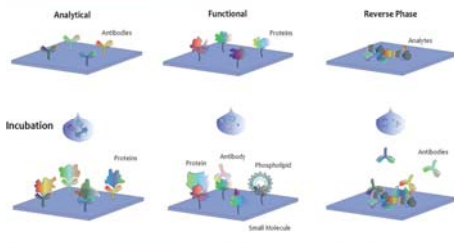


- The sample is prepared and incubated with the microarray.
- Previously, a corresponding binding partner for the molecule of interest was immobilized on the surface of the microarray. Here, an interacting antibody and antigen are depicted.
- Detection is performed by labeling a secondary antibody, which results in intensive signals if the molecule of interest is present.

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Abel, L. et al. Autoimmune profiling with protein microarrays in clinic applications. *Biochimica et Biophysica Acta* 1844 (2014) 977-987

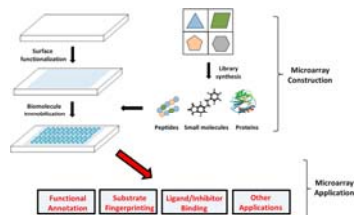
Three General Protein Microarray Types...



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Romanov, V. et al. A critical comparison of protein microarray fabrication technologies. *Analyst*, 2014, 139, 1303-1326.






Protein, Peptide and Small Molecule Array...



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Sun, Hongyan, et al. Recent advances in microarray technologies for proteomics. *Chemistry & Biology* 20, May 23, 2013.

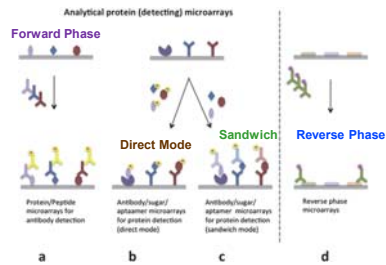
Capture Molecules

Type	Principle	Application
Protein-Protein		<ul style="list-style-type: none"> Disease progression Signal-pathway studies
Enzyme-Substrate		<ul style="list-style-type: none"> Substrate binding analyses
Receptor-Ligand		<ul style="list-style-type: none"> Drug discovery
Antigen-Antibody		<ul style="list-style-type: none"> Biomarker identification in auto-immune diseases
Aptamers		<ul style="list-style-type: none"> Protein-protein interaction analyses

Abel, L. et al. Autoimmune profiling with protein microarrays in clinic applications. *Biochimica et Biophysica Acta* 1844 (2014) 977-987

Protein Detecting Microarray...

Forward Phase (a)
 Proteins and peptides are immobilized as capturing agents for antibody detection, whereas an anti-immunoglobulin, a common labelled antibody, is used for detection.

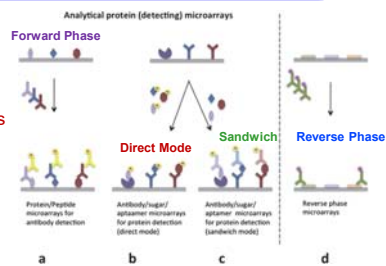


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Crelich, M. et al. Protein microarray technology: how far off is routine diagnostics? *Analyst*, 2014, 139, 528.

Protein Detecting Microarray...

Direct Mode (b)
 Antibodies, sugars, or aptamers are immobilized, and labelled proteins are captured.



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Crelich, M. et al. Protein microarray technology: how far off is routine diagnostics? *Analyst*, 2014, 139, 528.

Factors Affecting Performance...

- Appropriate surface for the immobilization of either protein or antibody samples.
- **Microarray patterning technique.**
- Protein conformational changes with expression, purification or immobilization may alter their function or render them inactive.
- Charged surfaces, temperature, pH and solvents may denature some proteins, and therefore surfaces must be biocompatible to minimize denaturation.
- Protein instability may lessen shelf-life.

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Summary

- DNA microarrays
 - Looking for gene mutations with DNA
 - Studying gene expression with mRNA
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 - Polymerase chain reaction (PCR)
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- Proteomics
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 - Examples of DNA lab-on-a-chip devices.

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Antibody Classes

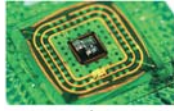
Antibody Class	Structure	Weight	Half-life	Function
IgG		~150 kDa	~21 days	Neutralization, opsonization, complement activation
IgM		~900 kDa	~5-10 days	Agglutination, complement activation
IgA		~350 kDa	~6-8 days	Mucosal immunity, neutralization
IgE		~185 kDa	~2-3 weeks	Allergic reactions, parasitic immunity

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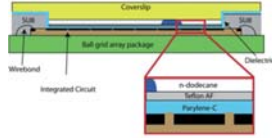
<https://www.novusbio.com/support/general-support/antibody-basics.html>

Fully Encapsulated PCR Chip...

a) Fully encapsulated PCR chip using SU8 to protect the wirebonds.



b) Sidewalls have gradient of 89.9° enabled with use of long-pass filter during UV exposure.



c) A special coverslip is used to compress the droplet and improve electrowetting droplet transport.

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Norian, H. et al., An integrated CMOS qualitative-polymerase-chain-reaction lab-on-a-chip for point-of-care diagnostics. *Lab Chip*, 2014, 14, 4076-4085

A PCR + Microarray Device – Petralia et. al.

The sample preparation process was entirely performed in a single step in the silicon microreactor, and assessed by RT-qPCR and direct DNA quantification.

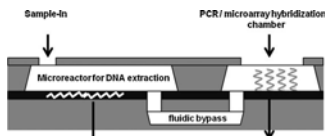
The extracted DNA, after PCR amplification, was hybridized on the DNA microarray.



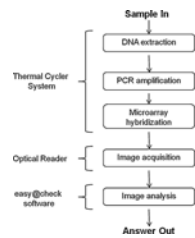
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Petralia, S et al., In-Check system: a highly integrated silicon lab-on-chip for sample preparation, PCR amplification and microarray detection of nucleic acids directly from biological samples. *Sensors and Actuators B* 187 (2013) 99-105.

Lab-on-a-Chip Cross-Section & Process...



Both the DNA amount and the amplification efficiency were higher than those obtained with a conventional multi-step process used for comparison.



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Petralia, S et al., In-Check system: a highly integrated silicon lab-on-chip for sample preparation, PCR amplification and microarray detection of nucleic acids directly from biological samples. *Sensors and Actuators B* 187 (2013) 99-105.

A miRNA Detector – Wang et. al.

- **MicroRNAs**
 - Class of *endogenous and noncoding* RNAs.
 - Crucial to gene regulation, such as cell development, differentiation, metabolism, and apoptosis.
 - **18-25 nucleotides** long, low content and easily degraded.
- **Photoelectrochemical (PEC)** biosensor for microRNA detection.
 - **MoS₂/g-C₃N₄/black TiO₂ ternary heterojunction** as the photoactive material and **gold nanoparticles** carrying **Histostar antibodies** (Histostar@AuNPs) for signal amplification.
 - Deposited on **indium tin oxide (ITO)** electrode.

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Wang MH, Yin HS, Zhou YL, et al. Photoelectrochemical biosensor for microRNA detection based on a MoS₂/g-C₃N₄/black TiO₂ heterojunction with Histostar@AuNPs for signal amplification. *Biosensors & Bioelectronics*. 2019;128:137-143.

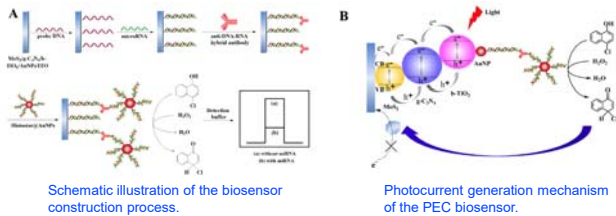
- **PEC biosensors** - low background noise, easy operation and excellent sensitivity.
- The challenge in PEC biosensors is *suppressing the recombination of photo-generated electrons and holes in semiconductor materials*.
 - Suppression will increase photo-excited carrier lifetimes.
- **Ternary heterojunctions** improve **light absorption efficiency, promote electron transfer** and **extend the lifetime of charge carriers**,

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Wang MH, Yin HS, Zhou YL, et al. Photoelectrochemical biosensor for microRNA detection based on a MoS₂/g-C₃N₄/black TiO₂ heterojunction with Histostar@AuNPs for signal amplification. *Biosensors & Bioelectronics*. 2019;128:137-143.

Construction and Photocurrent Generation...

Photoelectrochemical biosensor for microRNA detection

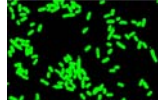


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Rapid Detection of Pathogens - Hügler et. al.

- Fluorescent *E. coli* were pre-concentrated from an initial volume of 1 ml down to 5 ml. Subsequent steps allowed for measurement of organism specific *sfGFP mRNA*.
- Pre-concentration was followed by thermoelectric lysis and on-chip gel-electrophoresis of released nucleic acids (including mRNAs) to remove larger fractions (e.g. gDNA or plasmids) and residual cell debris.
- Specific *sfGFP mRNA* was then quantified in the extracts by RT-qPCR.



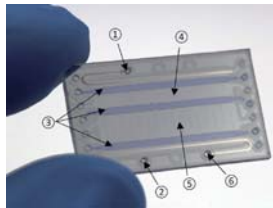
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Fluorescence image of *E. coli* courtesy of Flora V. Romeo.

Preconcentration and Nucleic Acid Extraction...

Microfluidic chip for preconcentration and lysis of bacteria as well as gel-electrophoresis of nucleic acids.

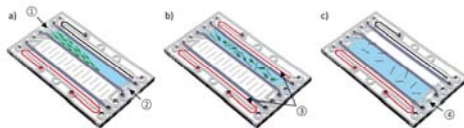
- 1) Cathode.
- 2) Anode.
- 3) Hydrogel .
- 4) Sample chamber.
- 5) Elution chamber.
- 6) Electrode test pin (not used).



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Hügler M, Dame G, Behrmann O, et al. A lab-on-a-chip for preconcentration of bacteria and nucleic acid extraction. *Rsc Advances*. 2018;8(36):20124-20130.

CAD Illustration of the Process Flow...



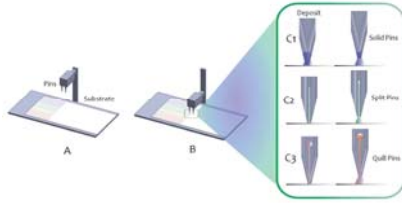
- a) Bacteria are captured between the anode (red) and cathode (black) at the middle gel front using free-flow electrophoresis ① inlet (sample) ② outlet (waste).
- b) A sinusoidal AC voltage is applied to cause lysis of the concentrated bacteria leading to a release of their genetic material ③ electrodes.
- c) Nucleic acids are transported through the middle gel into the elution chamber by gel-electrophoresis (anode: red, cathode: black). ④ Outlet.

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Pin Printing...

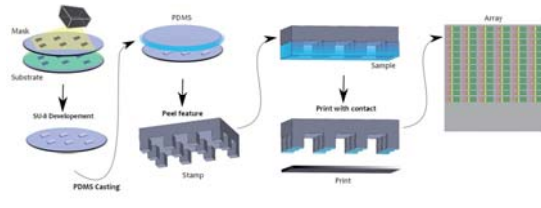
- a) A robotic print head with multiple printing pins is loaded with print solutions from a source plate and then contacts the substrate surface to deposit protein solution in
- b) Various types of pins: C1 is a solid pin, C2 is a slotted pin, C3 is a quill pin, distinguished from the split pin by the inclusion of a reservoir.



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Romanov, V. et al. A critical comparison of protein microarray fabrication technologies. *Analyst*, 2014, 139, 1303-1326.

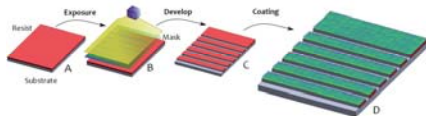
Microstamp Fabrication and Printing...



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Photolithographic...

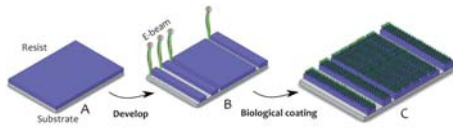


- a) Undeveloped photoresist (red).
 b) Photoresist is exposed to light (yellow) through the photomask.
 c) Development removes the exposed, softened photoresist and a nano-patterned photoresist is generated.
 d) Microarray is generated by attachment of proteins to patterned photoresist.

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E-Beam Lithography

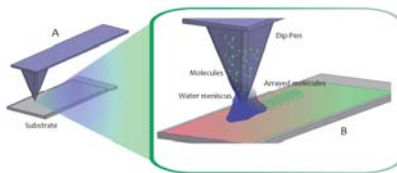


- Undeveloped photoresist.
- E-beam ablates photoresist.
- Protein/antibodies attach to photoresist to generate array.

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Dip Pen Nanolithography



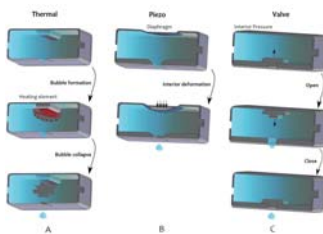
- Patterned microarray generated by AFM.
- Previously dipped AFM tip transferring protein solution to surface. A meniscus of protein solution on the AFM tip transports molecules to the surface in the desired pattern.

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Single Droplet Noncontact Printing...

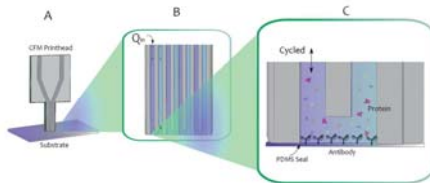
- Thermal Inkjet. A heating element rapidly creates a bubble within the chamber. As the bubble propagates, liquid is further squeezed out of the orifice. Upon bubble collapse, sample is ejected.
- Piezo actuation. A diaphragm is used to displace the sample within.
- Pressure valve.



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A Microfluidic-Interfaced Printer...



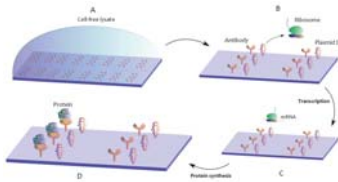
- CFM print head is docked against the surface.
- Close-up of the flow cells within the print head.
- Close-up of one channel. Solution can be cycled back and forth over the surface, ensuring total coverage of the surface.

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Nucleic Acid Programmable Protein Array...

- Expression plasmids encoding the proteins, as glutathione s-transferase (GST) fusions are biotinylated and immobilized onto a glass slide that has been coated with avidin and an anti-GST antibody which acts as the protein capture reagent.
- Plasmid array is then used for in situ expression of the proteins using rabbit reticulocyte cell lysate or a similar cell-free expression system.
- The protein is synthesized.
- The protein is immediately captured by the immobilized antibody within each spot. This process generates a protein array where every protein is co-localized with its analogous expression plasmid.

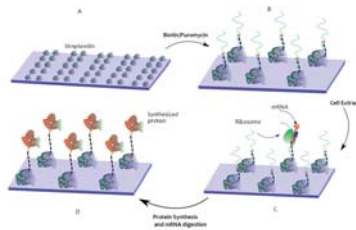


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In-Situ Puromycin Capture...

- A streptavidin surface.
- mRNA is hybridized with a single-stranded DNA oligonucleotide that has been modified with biotin and puromycin.
- The ribosome interacts with the RNA/DNA section of the molecule, where DNA is cross-linked to the nascent polypeptide through the puromycin moiety.
- mRNA is digested with added RNase, leaving a protein array immobilized through the C-termini to the DNA linker, which is in turn immobilized through a biotin/streptavidin interaction to the surface.

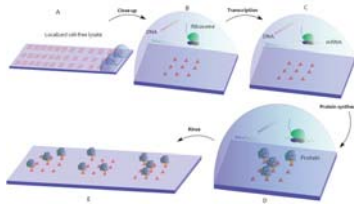


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Protein In-Situ Array...

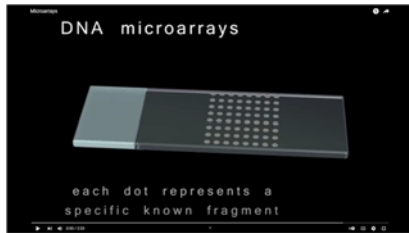
- Protein capture tags are array on the surface
- DNA and cell-free extract is added to the slide
- mRNA is produced via the cDNA template
- Newly synthesized protein is captured by the capture agent via a tag
- Slide is washed to remove any non-specific binding and is ready for quantification.



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DNA & Protein Microarrays Compared...



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<https://youtu.be/ePFE7yg7LvM>
