Cytokine analysis by multiplexed FlowMetrix.
Excitation and emission spectra of the orange and red fluorochromes were analyzed using single-color microspheres. The emission spectra were collected using 488 nm excitation. The excitation spectra were collected at emission wavelengths of 600 nm (orange) and 660 nm (red), respectively.


Microsphere fluorescence intensity bivariate plots. The emissions were split using a 50/50 beam splitter.

LP: Long Pass filter
SP: Short pass filter
Fig. 1. Cytokine analysis by multiplexed FlowMetrix E. (A) A pictorial representation of the reagents used in this assay. Blue wavy lines represent the excitation light beam, straight colored lines the emission beams. (B) Each cytokine assay is coupled to a bead population with discrete FL2 and FL3 fluorescence allowing separation by automatic gating (gray ovals indicate gates). Designation of cytokine assays to bead sets shown in right panel. Biotin indicates the bead set use as a positive control during calibration. (C) Comparison of the FlowMetrix E and ELISA assays. Cytokine standards were quantified using ELISA (green), individual FlowMetrix E (using a single standard and pair of anti-cytokine mAbs) (mauve) and multiplexed FlowMetrix E assays (using a combination of all the cytokine standards and anti-cytokine antibody pairs) (blue). Individual FlowMetrix E standards were pooled after staining with the detection antibody for simultaneous analysis. The limit of detection for the FlowMetrix E assay is defined by the upper 99% confidence limit of the background due to cross-reactivity with other assays (red). FlowMetrix E data are plotted against the left-hand scales, starting with the background of unstained beads. ELISA data are plotted against the right-hand scales, starting with the background due to substrate alone. Multiplexed assays were performed with all 15 cytokine assays; IL-4 and IFN-γ are shown as examples (Carson and Vignali, 1999).
Immunoassays 2

Hubert H. Girault - Niels Lion
Spring 2011
Immunoassays

Strategies to immobilize few antigens in solutions on surface bound antibodies.

- **Approach 1**: “Ticket control”
  - Force all the solution to pass through a narrow channel

- **Approach 2**: “Divide to rule”
  - Disperse the solid phase in solution
  - Adsorption in solution
  - Collection on the solid phase
Magnetic beads

Dynabeads® come in several sizes and with different surface functionalities, for use in a wide variety of applications.

Some beads are pre-coupled with a biomolecule (ligand). The ligand can be an antibody, protein or antigen, DNA/RNA probe or any other molecule with an affinity for the desired target.

1. **Bind**
   When added to a sample, Dynabeads® bind to the desired target (cells, pathogenic microorganisms, nucleic acids, peptide, protein or protein complex etc). This interaction relies on the specific affinity of the ligand on the surface of the beads.

2. **Wash**
   The beads respond to a magnetic field, allowing bound material to be rapidly and efficiently separated from the rest of the sample. Unbound material is simply removed by aspiration, and the bead-bound target washed by the use of the magnet.

3. **Elute**
   The bead-bound target is released in a suitable volume for use in downstream applications. Alternatively, the bead-bound target can be used directly while still attached to the beads.
Superparamagnetic microbeads

1 μm of diameter

Magnetisable microbeads

-~10^5 small iron oxide grains (approx. 8 - 15 nm)
-polymer (e.g. polystyrene)

No magnetic memory effect
Dynabeads® M-270 Streptavidin

**figure 2.** Monodisperse superparamagnetic 2.8 µm Dynabeads®

One mg of Dynabeads® M-270 Streptavidin typically binds:

- > 950 pmoles free biotin
- approx. 200 pmol biotinylated peptides
- approx. 10 µg biotinylated IgG
- approx. 10 µg ds-DNA
- approx. 200 pmol ss-Oligo nucleotides.

Each bead has an even dispersion of superparamagnetic material (γFe₂O₃ and Fe₃O₄) coated with a thin polymer shell to encase the magnetic material. This provides a specific and defined surface for coupling of target ligand. The true uniformity of all beads within each batch (typical CV<3%) provides consistent physical and chemical properties. Unique batch-to-batch reproducibility (typically < 5%) secures the reproducibility and quality of your results.
The Streptavidin Magnetic Particles are polydisperse paramagnetic particles that are designed for the fast separation of a variety of biotin-labeled molecules. The particles are supplied in a suspension that contains 10 mg particles per ml.

The magnetic separation of biotin-labeled molecules has been successfully employed in a wide range of applications.

Binding capacities per mg streptavidin magnetic particles:

- ≥1800 pmol free biotin
- >150 pmol biotin-labeled oligonucleotide
- >10 pmol biotin-labeled dsDNA fragment
Diffusion limited reaction

Reaction rate

\[ v_{AB} = 4\pi (D_A + D_B) d_{AB} N_{Av} [A][B] \]

Stokes Einstein equation

\[ D = \frac{kT}{6\pi \eta r} \]

\[ k_D = \frac{2RT}{3\eta} \frac{(r_A + r_B)^2}{r_A r_B} \approx \frac{2RT}{3\eta} \frac{r_A}{r_B} \text{ if } r_A >> r_B \]
Spherical geometry

Diffusion layer thickness = Bead Radius

\[ \theta << \theta_{eq} \]
\[ \Gamma(t) = \left( \frac{k_{on} c_{\text{bulk}} \Gamma_{\text{max}}}{1 + \frac{k_{on} \Gamma_{\text{max}} R}{D}} \right) t = \left( \frac{\Gamma_{eq}}{1 + Da} \right) \frac{t}{t_d} \]

\[ \psi << 1 \]
\[ \Gamma(t) = K \Gamma_{\text{max}} c_{\text{bulk}} \left[ 1 - \exp \left( -\left( \frac{D}{RK \Gamma_{\text{max}}} \right) t \right) \right] \]

\[ Da >> 1 \]
\[ \Gamma(t) = \Gamma_{eq} \left[ 1 - \exp \left( -\frac{1}{Da \left( \frac{t}{t_d} \right)} \right) \right] \]
Scaling beads

Calculate the mass of beads (Ø = 1 μm), the time and the capture efficiency to bind 100,000 molecules from a 0.1 pM solution. Sample volume = 100 μL

Is it diffusion or kinetically controlled?

\[ \Gamma_{\text{max}} \approx 10^{-9} \text{ mol} \cdot \text{m}^{-2} \]

\[ K = 10^9 \text{ M}^{-1} \]

\[ k_{\text{on}} = 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} \]

\[ c^{\text{bulk}} = 0.1 \text{ pM} \]

\[ D = 10^{-10} \text{ m}^2 \cdot \text{s}^{-1} \]
Elecsys - Roche
Magnetic beads + Conjugates

Analyte + ECL tagged Antibody → Magnetic bead Capture Antibody
Electrochemiluminescence

Radical annihilation

Reduction \[ R + e^- \rightarrow R^- \] \[ E_{R/R^-}^{\ominus} \]

Oxidation \[ R - e^- \rightarrow R^+ \] \[ E_{R^+/R}^{\ominus} \]

Radical annihilation \[ R^- + R^+ \rightarrow 1R^* + R \]

Emission \[ 1R^* \rightarrow R + h\nu \]

\[ R = Ru(bpy)_3^{2+} \] Emission at 610 nm
Electrochemiluminescence

Using different precursors

Reduction: $A + e^- \rightarrow A^{\cdot-}$
Oxidation: $D - e^- \rightarrow D^{\cdot+}$

Radical annihilation: $A^{\cdot-} + D^{\cdot+} \rightarrow {}^1A^* + D$
$\rightarrow {}^1D^* + A$

Emission: $^1A^* \rightarrow A + h\nu$
$^1D^* \rightarrow D + h\nu$

$E_{R/R^-}^\ominus$
$E_{R^+/R}^\ominus$
Electrochemiluminescence

\[ \Delta G / eV \]

- S-route: \[ R^- + R^+ \]
- T-routes:
  - \[ R^- + \text{TMPD}^+ \]
  - \[ R^+ + \text{BQ}^- \]

- Triplet-triplet annihilation:
  - \[ ^1R^* + R \]
  - \[ ^3R^* + R \]

R = Rubrene, TMPD = \( N,N,N',N' \)-tetramethyl-\( p \)-phenylenediamine, BQ = \( p \)-benzoquinone
ECL cell

Detection

Magnet

Solution + beads

Working electrode

Detection

Magnet
**Ru(bpy)$_3^{2+}$ Electrochemiluminescence**

**Fig. 1. Mechanism of ECL excitation**

Ru(bpy)$_3^{2+}$ and TPA are oxidized at the surface of a gold electrode, forming Ru(bpy)$_3^{3+}$ and TPA$^{++}$, respectively. The TPA$^{++}$ spontaneously loses a proton, forming TPA$^*$. The TPA$^*$, a strong reductant, reacts with Ru(bpy)$_3^{3+}$, a strong oxidant, forming the excited state of the label, Ru(bpy)$_3^{2+*}$. The excited state decays to the ground state through a normal fluorescence mechanism, emitting a photon having a wavelength of 620 nm.

**Fig. 3. electrochemical excitation waveforms and the resulting current and luminescence waveforms**

The potential is applied to the counterelectrode and the waveform shown is measured at the Ag/AgCl reference electrode. The current is measured at the working electrode, and the analytical signal (luminescence) is measured with the PMT mounted adjacent to the flow cell, as shown in Fig. 2.
Electrochemiluminescence detection uses labels that emit light when electrochemically stimulated. Background signals are minimal because the stimulation mechanism (electricity) is decoupled from the signal (light).

Labels are stable, non-radioactive and offer a choice of convenient coupling chemistries. They emit light at $\sim$620 nm, eliminating problems with color quenching. Few compounds interfere with electrochemiluminescent labels so you can use large, diverse libraries with confidence.

Multiple excitation cycles of each label amplify the signal to enhance light levels and improve sensitivity.
Potential dependence

The ECL emission as a function of potential for the Ru(bpy)$_3^{2+}$/TPrA system consists of two waves. The first occurs with the direct oxidation of TPrA at the electrode, and the second where Ru(bpy)$_3^{2+}$ is oxidized. In dilute Ru(bpy)$_3^{2+}$ solutions (<uM) containing mM TPrA, the intensity of the first ECL wave is significant and can be larger than that for the second ECL wave.

(a) ECL and (b) cyclic voltamogram of 1.0 nM Ru(bpy)$_3^{2+}$ in the presence of 0.10 M TPrA with 0.10 M Tris/0.10 M LiClO$_4$ buffer (pH 8) at a 3 mm diameter glassy carbon electrode at a scan rate of 50 mV/s. (c) As (a) but with 1.0 mM Ru(bpy)$_3^{2+}$. The ECL intensity scale is given for (c) and should be multiplied by 100 for (a).
A more complicated story...

Scheme 1

Scheme 2

Scheme 3
Elecsys 2010

The Elecsys 2010 provides automated immunodiagnostic testing with the ease-of-use and workflow flexibility of automated clinical chemistry systems, allowing for individualized, customer-specific solutions. Engineered for continuous random-access operation and stat capabilities, the Elecsys 2010 offers risk-free consolidation of multiple workstations for laboratories that seek to maximize productivity and efficiency. Sampling systems are available in either disk configuration or the Roche universal rack configuration. The universal rack enables integration with pre-analytical automation modules, clinical chemistry systems and/or additional Elecsys systems.

Features

- Liquid, ready-to-use reagents
- Intuitive software
- Innovative two-dimensional barcode technology for reagents, calibrators and controls
- Assays with 9-, 18- and 27-minute incubation
- Electrochemiluminescent technology
- Choice of disk or rack sampling

Specifications

Throughput: Up to 86 determinations per hour
Tests channels: Up to 15 tests online
Technology: Electrochemiluminescence
Sample capacity/positions: Elecsys 2010 disk has 30 positions for samples Elecsys 2010 rack has 100 positions for samples
Sample volume/containers: Accepts both barcoded primary tubes and sample cups
Chemistry application overview: Thyroid, cardiac, anaemia, hepatitis, fertility, tumor markers, osteoporosis
Magnetism

- \( B \): magnetic induction [T]
- \( H \): magnetic field [A/m]
- \( M \): magnetization [A/m]
- \( \mu \): permeability [H/m]
- \( \mu_r \): relative permeability [-]
- \( \chi \): magnetic susceptibility [-]
- \( \phi \): scalar magnetic potential [A]
- \( F \): magnetic force [N]

\[
B = \mu_0 H
\]

\[
B = \mu_0 (H + M) = \mu_0 (1 + \chi) H = \mu_0 \mu_r H = \mu H
\]

\[
H = -\nabla \phi
\]

\[
F_{mag} = \nabla (m_{bead} \cdot B) \approx (m_{bead} \cdot \nabla) B
\]

\[
F_{mag} = \frac{V \Delta \chi}{\mu_0} (B \cdot \nabla) B
\]

\[
F_x = B_x \frac{\partial B_x}{\partial x} - B_y \frac{\partial B_x}{\partial y}
\]

\[
F_y = B_x \frac{\partial B_y}{\partial x} - B_y \frac{\partial B_y}{\partial y}
\]
How to place magnets?

Attraction

Repulsion

One Magnet

\[ l = 200 \, \mu m \]
\[ h = 200 \, \mu m \]
\[ s = 200 \, \mu m \]
Magnetic fields

Attraction

Repulsion

Vector representation

One magnet
Magnetic forces

\[ F_{\text{mag}} = \frac{V \Delta \chi}{\mu_0} (B \cdot \nabla) B \]
Bead trapping in a capillary

(a) Magnetic force (x component) calculated for a bead of diameter=1 μm and χ=1. The values are taken along a horizontal path (the x axis). (A) attraction, (B) repulsion and (C) single magnet. The vertical dashed lines show the magnets position.

Microscopic visualizations of the MBs plug in a capillary according to the magnets configurations. (b) attraction, (c) repulsion, (d) one magnet. h=8 mm, l=2mm and s=1 mm. capillary i.d.=100 μm.
Bead ELISA in a microchannel

**Immobilize bead- antibody**

**Blocking step**

**Diffusion controlled antigen adsorption**

**Enzyme labelled secondary antibody**

**Add substrate**

**Incubate**

**Amperometry**

*Dimension: 50 μm · 100 μm · 1 cm*

*Volume: 50 nL*

*Surface area = 3 mm²*

*Surf/Vol = 60'000 m⁻¹*
ELISA in a microchannel

1. Add substrate
2. Incubate
3. Amperometry

Magnetic bead with antibody+antigen+label

Dimension: 50 μm · 100 μm · 1 cm
Volume: 50 nL
Scaling in bio-analytics

Sample volume / L

Concentration / M

Electrochemical methods

Fluorescence

Mass spectrometry

Microfluidics

Immunoassay range

Proteomics range

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Wednesday, March 9, 2011
**γ-Interferon detection (IFN-γ) by GRAVI™-Cell ELISA**

This application note delineates speed and sample volume benefits of GRAVI™-Cell in the detection of IFN-γ. The assay is performed with magnetic particles as solid support, and a set of sandwich antibodies for the specific capture and detection of IFN-γ; whilst streptavidin-alkaline phosphatase conjugate (strep-ALP) mediates readout by electrochemical signal detection in the GRAVI™-Chip.

**Assay principle**

- **Materials**

  This IFN-γ assay was performed with the GRAVI™-Cell & GRAVI™-Chip platform, using p-aminophenyl phosphate (PAPP) enzyme substrate from DiagnoSwiss (Ref. IP-010-00, CG-010-01 and resp. RS-001-50), tosylactivated beads from Dynal (MyOne™ Dynabeads Ref. 655.01), ELISA reagents from Biosource (IFN-γ kit containing capture antibody, standards and biotinylated secondary antibody, Ref. CHC1234), streptavidin-alkaline phosphatase conjugate from Biospa (Ref. SF0-61), and Dimethyl sulfoxide (DMSO) from Sigma-Aldrich (Ref. 34869) as regeneration solution.

  **Bead coupling with anti-IFN-γ antibodies (overnight)**

  The test makes use of capture antibody-coupled magnetic beads. Coupling is performed by mixture of 10µl capture antibody (2mg/ml) with 5µl of tosylactivated beads (100mg/ml) in sodium borate coating buffer during 15 hours at RT. Upon this simple coupling step, coated beads are washed as per recommended protocol (yielding volumes sufficient to obtain 250 data points in GRAVI™-Chips).
Interferon detection

Assay procedure (30 min incubation in tube + 10 min injection, washing, detection)

- Mix 20µl antibody-coated beads at 0.25mg/ml with 20µl sample or IFN-γ standard, 5µl biotinylated secondary antibody (4µg/ml) and 5µl streptavidin-ALP (1/100).
- Incubate on shaker for 30 minutes in tube at room temperature.
- Heat the GRAVI™-Cell to 37°C and prime a BSA-blocked GRAVI™-Chip through capillarity by depositing washing buffer (PBS buffer with 0.1% BSA and 0.1% Tween® 20) in the bottom reservoirs.
- Pipette 20µl of the incubated mix in the top reservoirs of the GRAVI™-Chip, for gravity-driven sample flow through the chip, and bead trapping nearby the electrodes by virtue of a magnet array.
- Within approx 7 min, most of the solution has passed through the chip. Proceed to wash the top reservoirs 3 x with 20µl substrate solution (PAPP @ 5mM at pH 10).
- Remove excess substrate from the top reservoirs, and start the one-minute enzyme detection by GRAVI™-Soft.
- Chip regeneration is performed by removing the magnets and pipetting 10µl DMSO in the top reservoirs; the chip is ready for a next experiment after 3 washing buffer steps.

Typical results

- The response is linear over the full range of detection: from 31 to 2000 pg/ml. The LOD (3x st.dev. @ 0pg/ml) is 5 pg/ml.
- CV in the medium to large concentration is typically under 10%.
- This IFN-γ assay using GRAVI™-Cell is performed in 40 min., as opposed to roughly 4 to 6 hours required for running standard micro-plate protocols.
- The sample volume is reduced to 10-20µl/data point.
- Cost of biological reagents and beads ~0.10 USD per data point.

References

Dip Pen Nanolithography® (DPN) is an established method of nanofabrication in which materials are deposited onto a surface via a sharp probe tip. Molecules are transferred from the tip to the surface through a water meniscus which forms in ambient conditions as the tip nears the surface.

DPN enables controlled deposition of a variety of nanoscale materials onto many different substrates. The vehicle for deposition can include pyramidal scanning probe microscope tips, hollow tips, and even tips on thermally actuated cantilevers. Recent advances have demonstrated scalability of the technique with arrays of tips leading to true massive parallelization with up to 55,000 tips.

The controlled transfer of a molecular 'ink' from a coated scanning force microscope tip to a substrate was described and initially developed by a research group at Northwestern University led by Professor Chad Mirkin. Analogous to the macro technique of a quill pen, these authors introduced the term Dip Pen Nanolithography or DPN to describe their work.
Nanoink