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Lab On a Chip:

Cell Manipulation: Cytometer

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Flow Cytometry

- Technology that measures properties of single cells
- Measures fluorescence, light scatter, and other properties of cells and particles
- Can provide correlated data that links different population profiles

What can Flow Cytometry Do?

- The most basic operation with cells is to count them.
- Flow cytometers are the devices that allow for counting particles suspended in a stream of fluid.
- Determine “biologicals” from “non-biologicals”
- Separate “live” from “dead” particles
- Evaluate 10^5 to 5×10^6 particles in less than 1 min
- Measure particle-scatter as well as innate fluorescence or 2° fluorescence
- Sort single particles for subsequent analysis

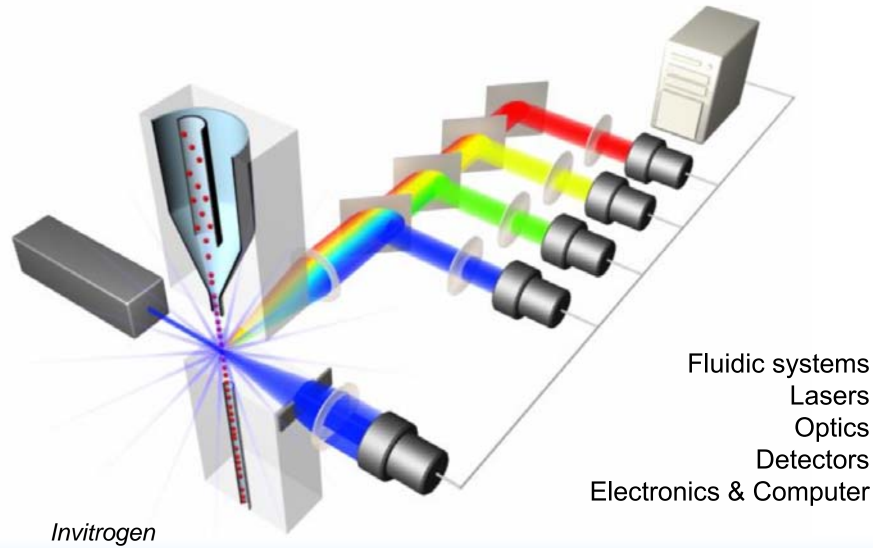
Flow Cytometer

- 流式細胞儀（flow cytometer）是現代生物學研究不可或缺的利器，除了可鑑定細胞的標記外，還可分析細胞的分裂週期、DNA 含量、細胞凋零死亡（apoptosis）、細胞內鈣離子的濃度變化、pH 值之改變……等等。此外，流式細胞儀更可將細胞篩選出來，供進一步研究，因此瞭解流式細胞儀的原理及用途，可為將來研究發展提供一個強而有力的利器
- Flow cytometers are expensive, bulky and tedious to operate – great interest to miniaturize and automate them.

Dr. 謝世良 @國立陽明大學 免疫學研究中心 主任

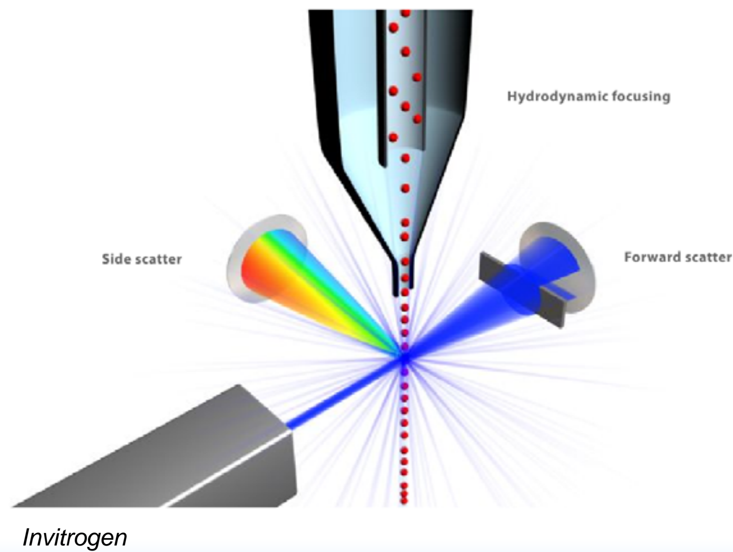
http://probes.invitrogen.com/resources/education/tutorials/4Intro_Flow/player.html

Cytometry Basic



Invitrogen

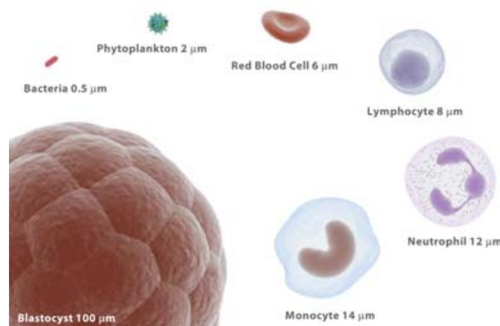
Interrogation Point



Invitrogen

Hydrodynamic Focusing and Size

- Particle or cells are passed through the laser beam one at a time.
- Flow cytometers can accommodate cells that span roughly three orders of magnitude in size.

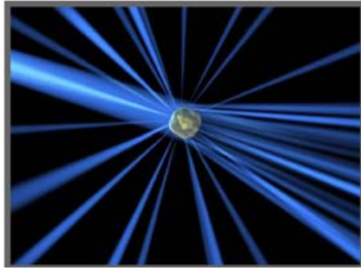


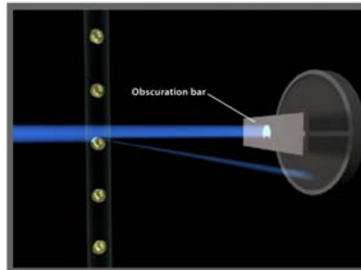
Invitrogen

淋巴细胞（Lymphocyte）

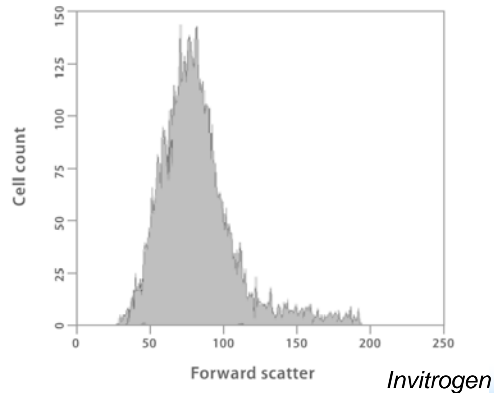
- 【正常参考值】百分值：0.20～0.40或（20%～40%）绝对值：0.8～4×109/L
- 淋巴细胞增高→ 病理性增高：
  - 感染性疾病，主要为病毒感染，如麻疹、风疹、水痘、流行性腮腺炎、传染性单核细胞增多症、传染性淋巴细胞增多症、病毒性肝炎、流行性出血热等。也可见于百日杆菌、结核杆菌、布氏杆菌、梅毒螺旋体等感染时。
  - 淋巴细胞性白血病、白血病性淋巴肉瘤。
  - 急性传染病的恢复期。
  - 组织移植后的排斥反应。
  - 其他：在再生障碍性贫血、粒细胞减少症和粒细.

Forward Scatter (Histogram)



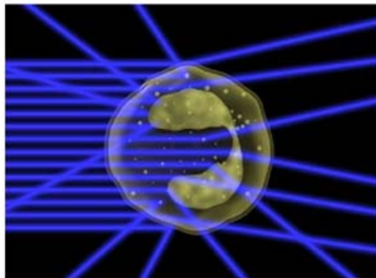


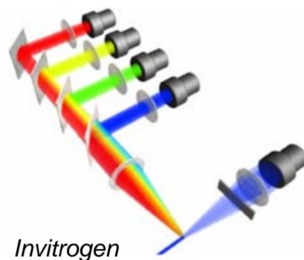
- Forward scatter (low-angle light scatter)
- Its magnitude is proportional to the size of the cell



Invitrogen

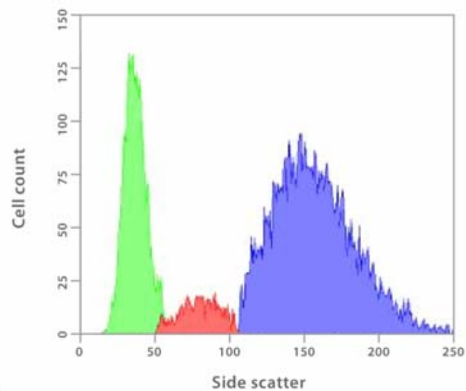
Side Scatter (Histogram)

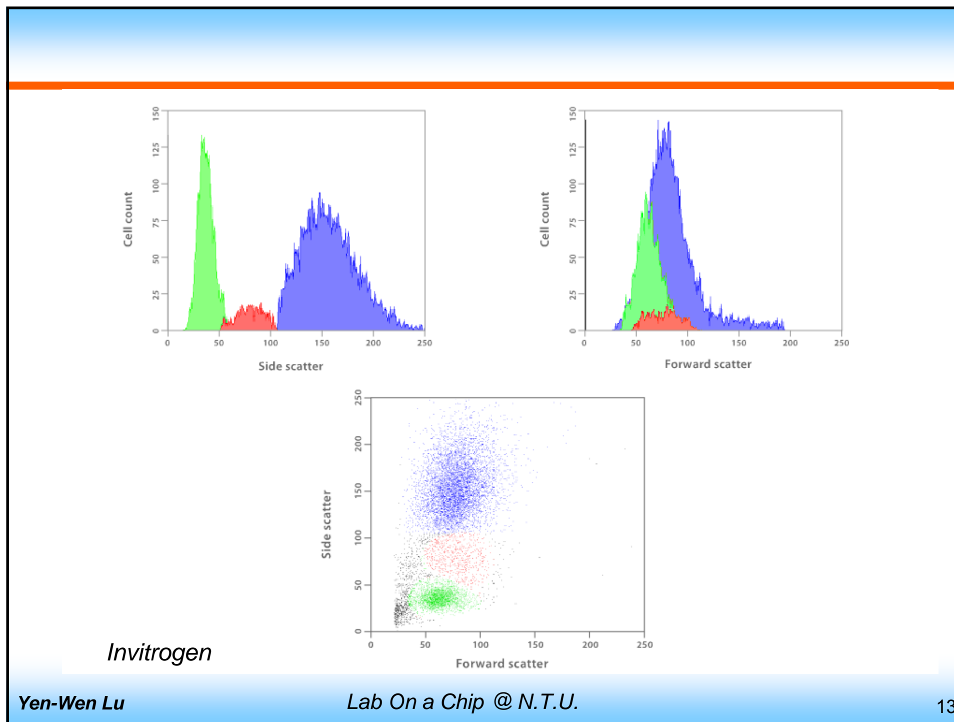




Invitrogen

- Side scatter is caused by granularity and structural complexity inside the cell




Cell count
Side scatter
Cell count
Forward scatter
Side scatter
Forward scatter
Invitrogen
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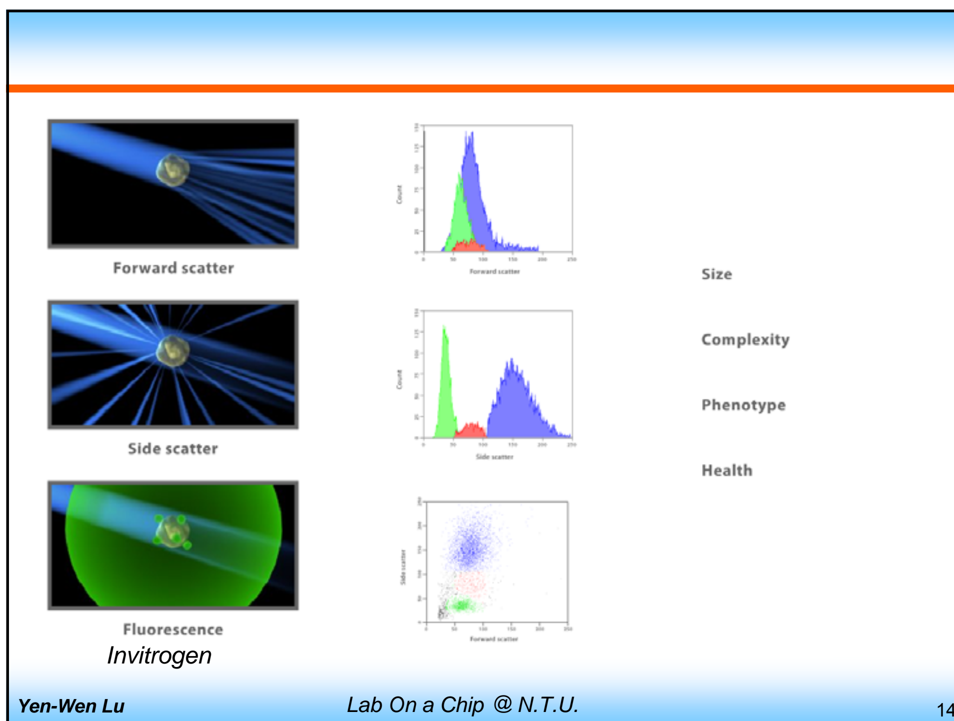
Forward scatter
Side scatter
Fluorescence
Invitrogen
Size
Complexity
Phenotype
Health
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Forward & Side Scatter

- Forward Scatter (FSC): Diffracted light
  - FSC is related to cell surface area (at narrow angles)
  - FSC is detected along axis of incident light in the forward direction
  - BUT – the accuracy for cell sizing is not good – it is very dependent upon the exact angle of scatter which can vary even between similar instruments
- Side Scatter (SSC): Reflected and Refracted light
  - SSC is related to cell granularity and complexity
  - SSC is detected (mostly) at 90° to the laser beam
- Factors that affect light scatter
  - Shape, surface, size, granularity, internal complexity, refractive index.

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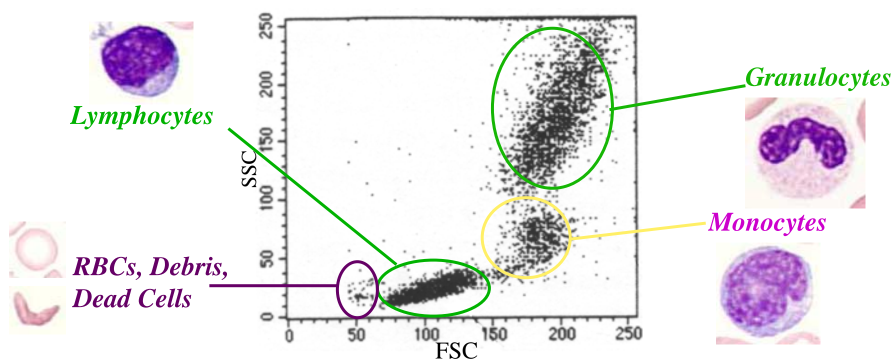
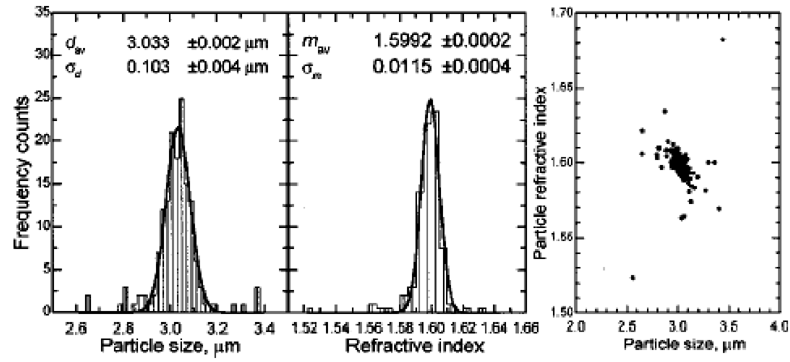
Why look at FSC vs SSC

Since FSC ~ size and SSC ~ internal structure, a correlated measurement between them provides a basis for a simple differentiation between the major populations



Applications - Polymer particles

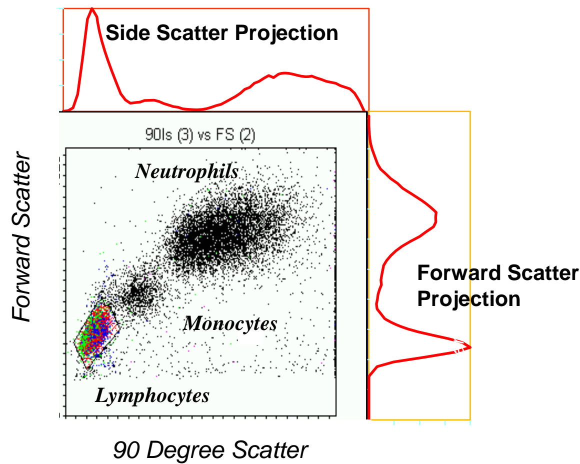
✓ **Particle size and refractive index distribution**



The polystyrene particles certified with the scanning flow cytometer

Review of Scientific Instruments, 71 (2000) 243~255

White blood cells



Light Scatter Gating

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Definitions

- **Flow Cytometry**
  - Measuring properties of cells in flow
- **Flow Sorting**
  - Sorting (separating) cells based on properties measured in flow
  - Also called **Fluorescence-Activated Cell Sorting (FACS) –** but this term does refer to sorting, not analysis. FACS is frequently misused. Saying FACS when you mean flow cytometry is incorrect!!

- Break Time !

Micro Cytometer

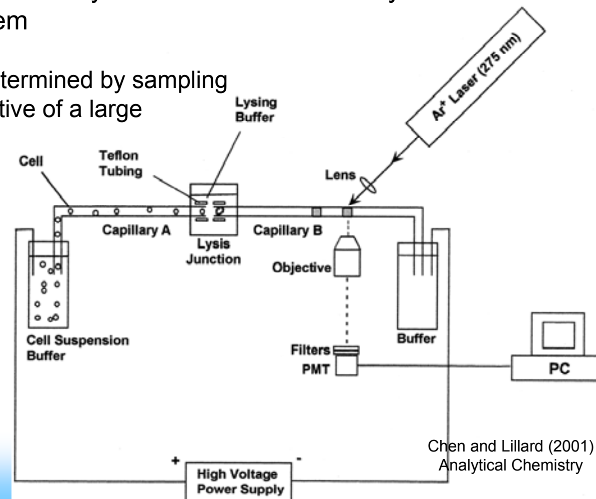
- Hydrodynamic focusing and optical detection systems make convectional cytometers complex, large and expensive, which require
  - Flow rate control on sheath and sample flows
  - Reservoir for the sheath flow medium
- Efforts to miniaturize a flow cytometer can be categorized into three different groups:
  - Microfluidic systems
  - Integration of a detection system
  - Cell sorter and counter

Single Cell Analysis by capillary EOF.

The cells were injected by EOF through the first capillary to a cell lysis junction. The resulting cell lysate migrated into the second capillary, where the major proteins, hemoglobin and carbonic anhydrase were separated by capillary electrophoresis and resultant analyte zone were detected by laser-induced fluorescent detection system

intracellular chemistry ➔ determined by sampling an aliquot that is representative of a large population of cells

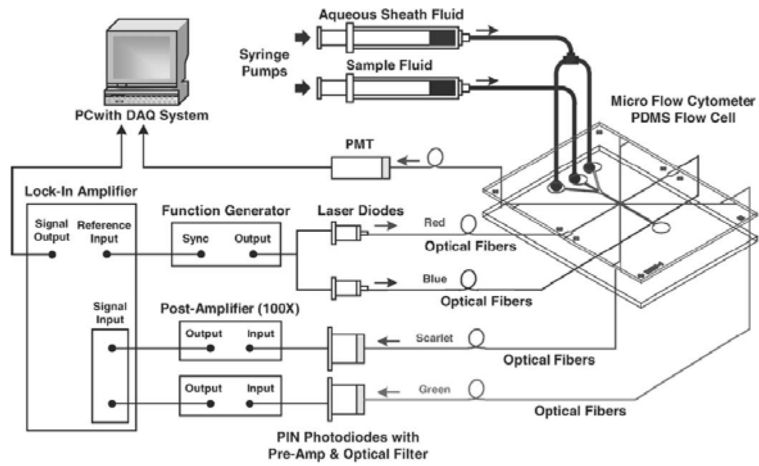
EOF?
Optical trapping?
Hydrodynamic?
Electrokinetics



Chen and Lillard (2001)
Analytical Chemistry

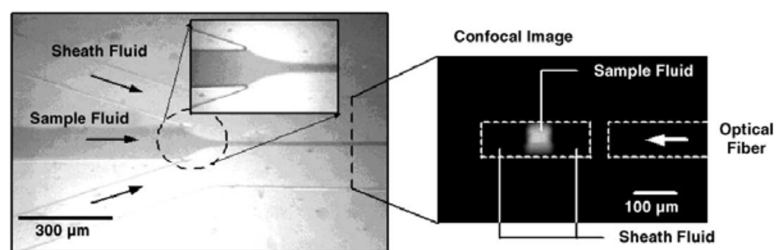
Figure 8 (online color at: www.biophotonics-journal.org) Early prototype of a microfluidic cytometry chip including waveguides and lenses for an excitation source (EX), a forward scatter collection line (FS) including a beam stop (BS), a side scatter collection line (SS), a large-angle scatter collection line (LAS), and a line for fluorescence collection (FL) (unpublished). The seamless integration of fluidic and photonic elements is accomplished by simple microfabrication techniques [60].

Detection Systems



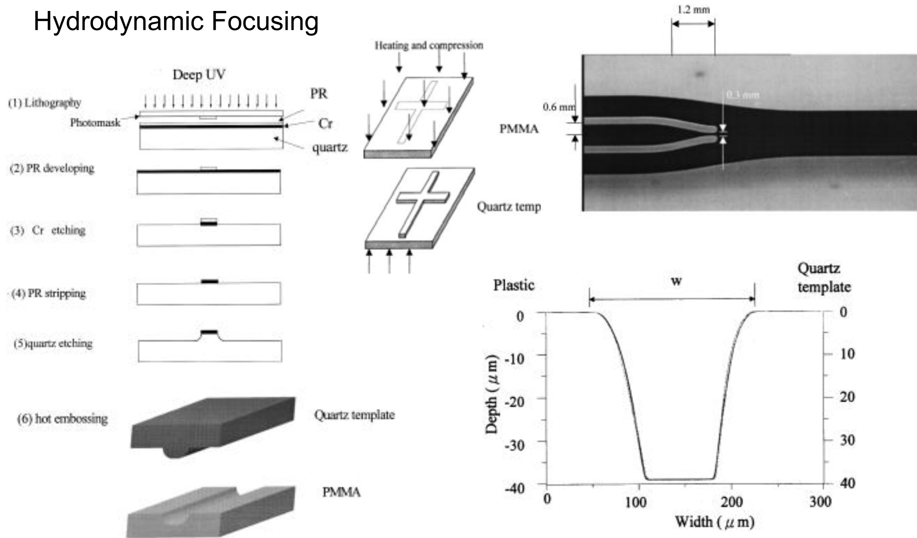
YC Tung et al, SNA B 2004
(林致廷 & Katsuo Kurabayashi)

Detection System



Particle Focusing Systems

Hydrodynamic Focusing



G. B. Lee et. al "Hydrodynamic Focusing for a Micromachined Flow Cytometer" J. Fluids Eng. 2001

Particle Focusing Systems

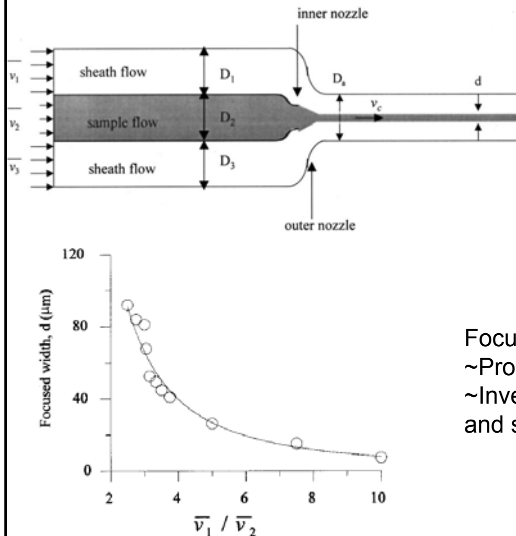
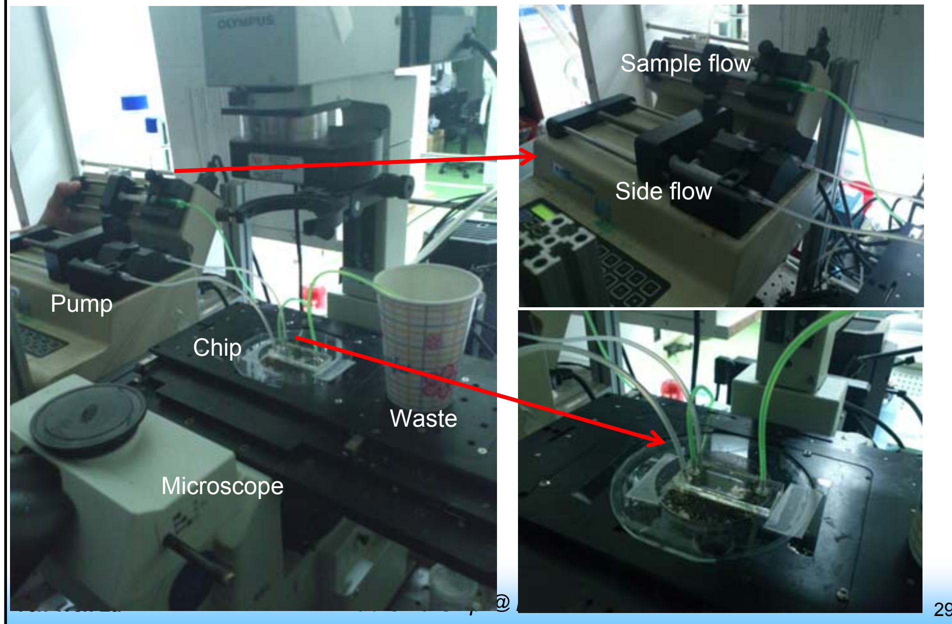


Fig. 10 The variation of the width of the focused stream along with relative flow rates while measured at the exit of the flow cytometer

$$d = \frac{\rho_a D_a}{1.5\left(\rho_1 \frac{v_1}{v_2}\frac{D_1}{D_2} + \rho_2 + \rho_3 \frac{v_3}{v_2}\frac{D_3}{D_2}\right)}$$

Focused stream width:
~Proportional to width of the outlet channel
~Inverse proportional to the relative sheath and sample flow rate

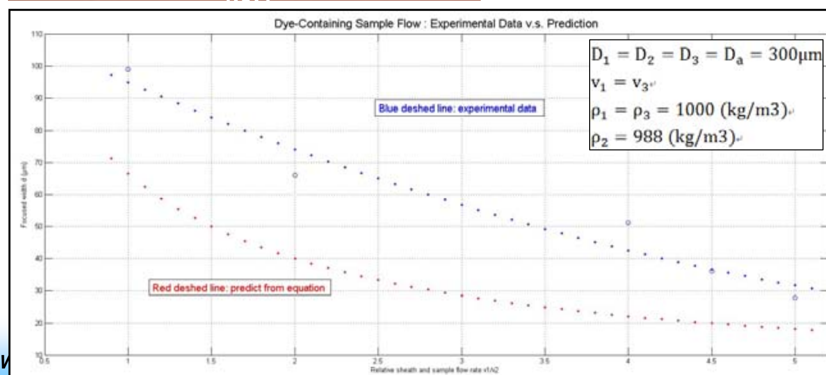
Experimental Setup



Trouble-Shooting

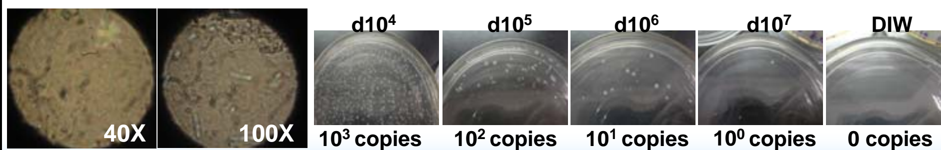
sample flow : side flow

Particle interference



Our Sample Target

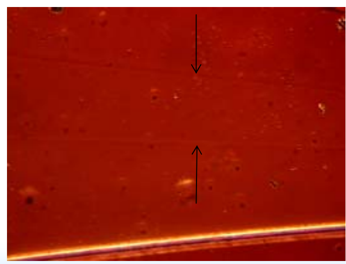
- Role of E-coli in Therapeutic applications and Biotechnology
  - Recombinant DNA technology was the manipulation of *E. coli* to produce human insulin.
  - The inability of *E. coli* to grow in water, combined with its short survival time in water environments, means that the detection of *E. coli* in a water system is a good indicator of recent faecal contamination.
  - Nonpathogenic *Escherichia coli* strain is used as a probiotic agent in medicine, mainly for the treatment of various gastroenterological diseases, including inflammatory bowel disease.

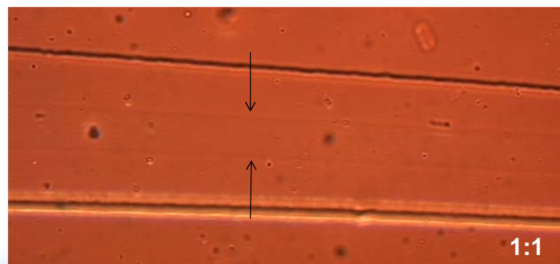


E. coli cytometry experiment

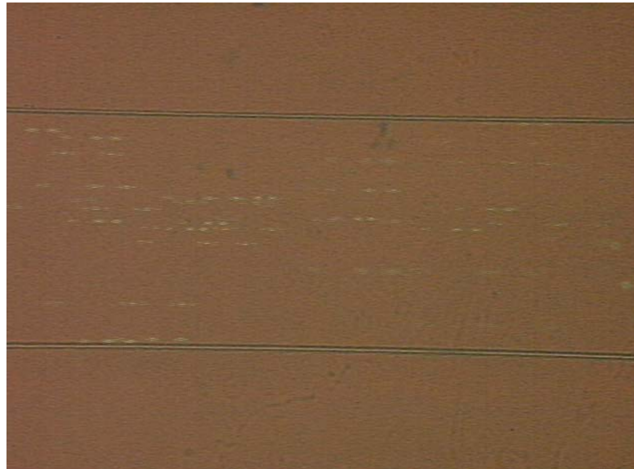
Microscope image of *E. coli*

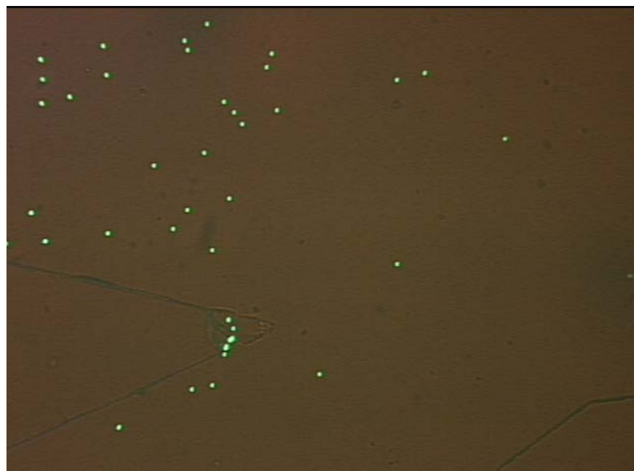
Microscope image of *E. coli* in channel





4.8 μm Green Fluorescent Polymer Microspheperes

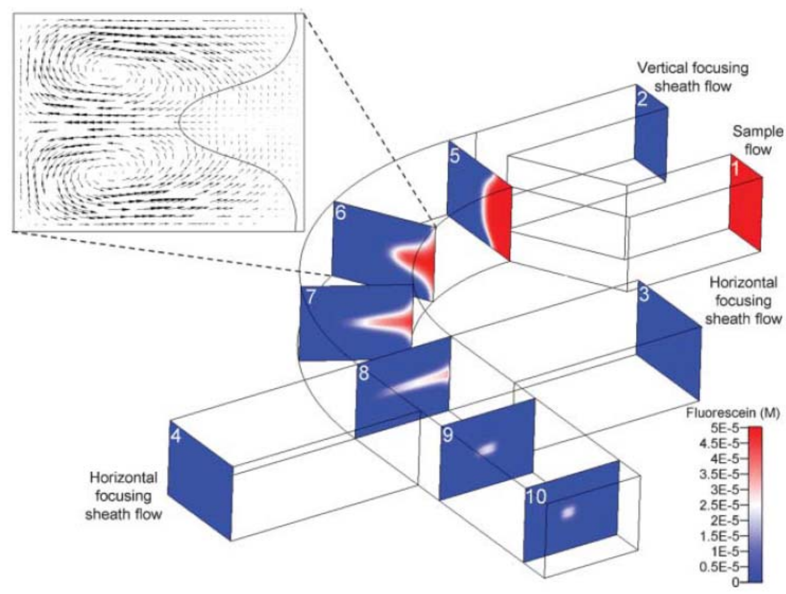




Summary

- The narrowest focusing flow can be achieved by applying the side flow and sample flow ratio up to 5:1.
- *E. coli* is small; they can be observed on static slide under microscope, but they are hard to be seen in flow.
- 4.8 μm Green Fluorescent Polymer Microspheperes showed more clear particle image in the microfluidic channel.

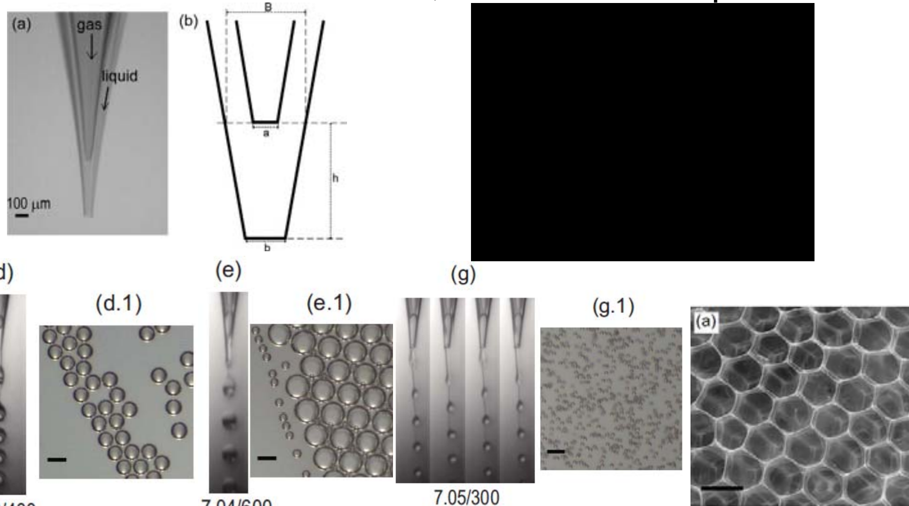
3D Focusing



- Is there any other way to control cells/particles?
- Hydrodynamic focusing?
- Microfluidics for particles
  - DEP?
  - Optical tweezers?

Hydrodynamic Focusing

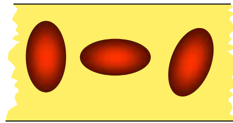
- Before we move further, one more example:

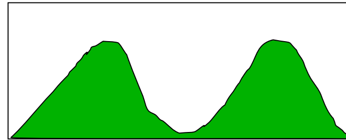


林耿慧. 中研院

History

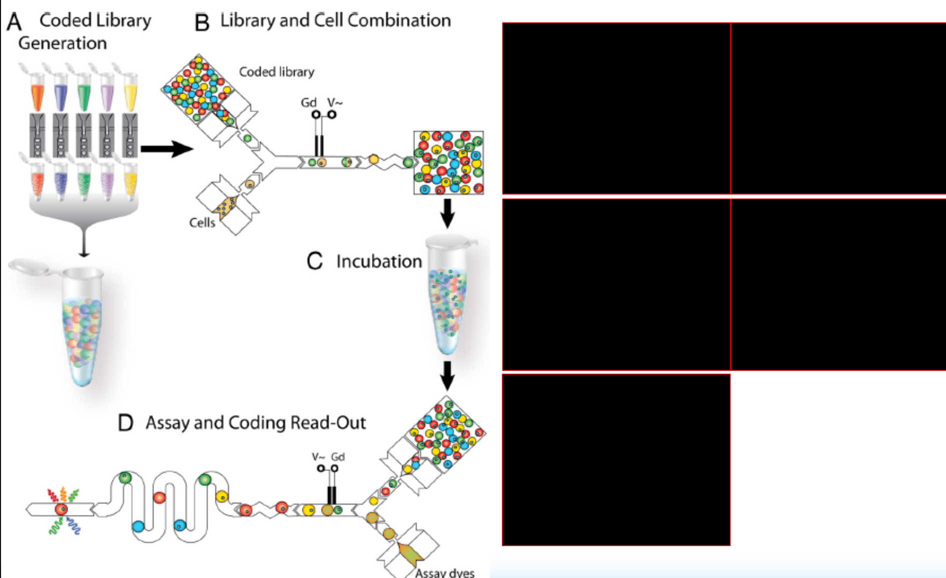
Mack Fulwyler – worked in Marvin van Dilla's lab at Los Alamos. – developed the sorter in 1965 – initially used electronic cell volume - at Los Alamos National Labs - this instrument separated cells based on electronic cell volume (same principle as the Coulter counter) and used electrostatic deflection to sort. The cells sorted were RBC because they observed a bimodal distribution of cell volume when counting cells - the sorting principle was based on that developed for the inkjet printer by Richard Sweet at Stanford in 1965.



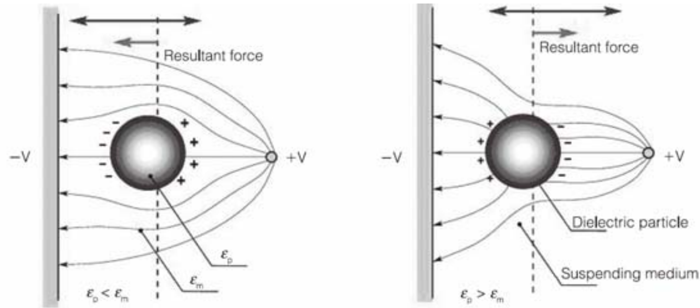


Electronic Cell Volume

Microdroplet Cultures and Assays



Revisit DEP

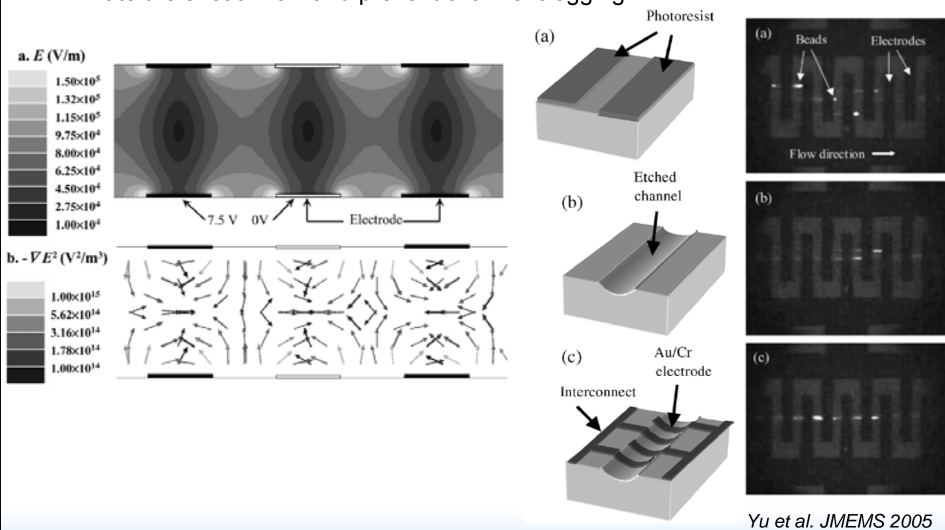


$$\vec{F}_{DEP}(x, y, z, \omega) = 2\pi\varepsilon_0\varepsilon_m R^3 \Re\{f_{CM}(\omega)\}\vec{\nabla}E^2_{rms}$$

where ε_0 is the vacuum dielectric constant, ε_m is the medium dielectric constant, R is the particle radius, E_{rms} is the root-mean-square value of the electric field, ω is the angular frequency, $\Re$ is the real part, and $f_{CM}(\omega)$ is the Clausius-Mossotti factor. The latter is a function of the complex permittivity of the particle and medium, and is defined as

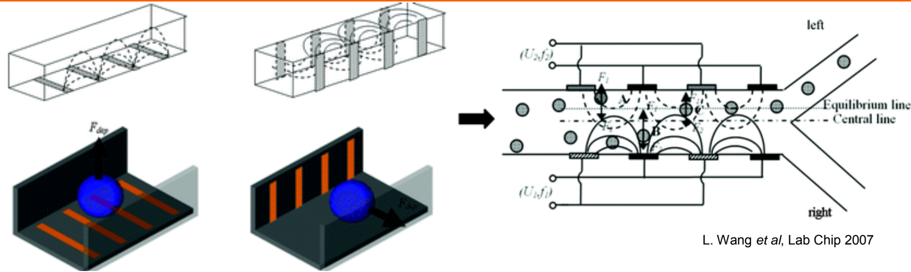
DEP Focusing

Eliminate the sheath flow and prevent channel clogging



Yu et al. JMEMS 2005

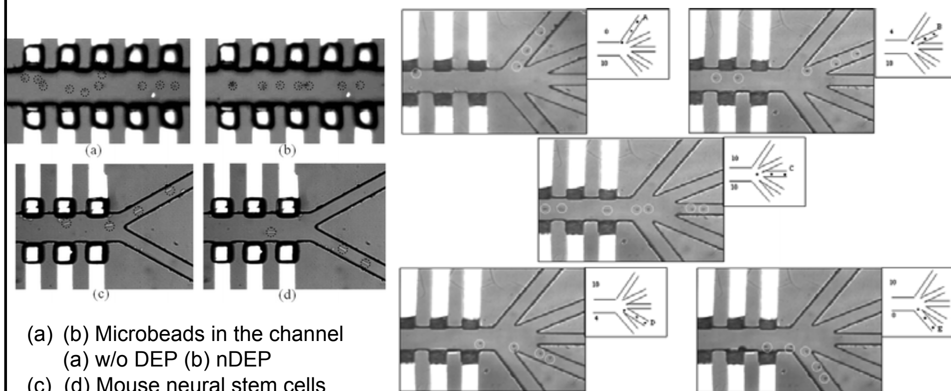
Vertical DEP for Flow Cytometry



L. Wang *et al*, Lab Chip 2007

A novel dielectrophoresis switching with vertical electrodes in the sidewall of microchannels for multiplexed switching of objects has been designed, fabricated and tested. With appropriate electrode design, lateral DEP force can be generated so that one can dynamically position particulates along the width of the channel. A set of interdigitated electrodes in the sidewall of the microchannels is used for the generation of non-uniform electrical fields to generate negative DEP forces that repel beads/cells from the sidewalls. A countering DEP force is generated from another set of electrodes patterned on the opposing sidewall. These lateral negative DEP forces can be adjusted by the voltage and frequency applied. By manipulating the coupled DEP forces, the particles flowing through the microchannel can be positioned at different equilibrium points along the width direction and continue to flow into different outlet channels. Experimental results for switching biological cells and polystyrene microbeads to multiple outlets (up to 5) have been achieved. This novel particle switching technique can be integrated with other particle detection components to enable microfluidic flow cytometry systems.

Vertical DEP for Flow Cytometry



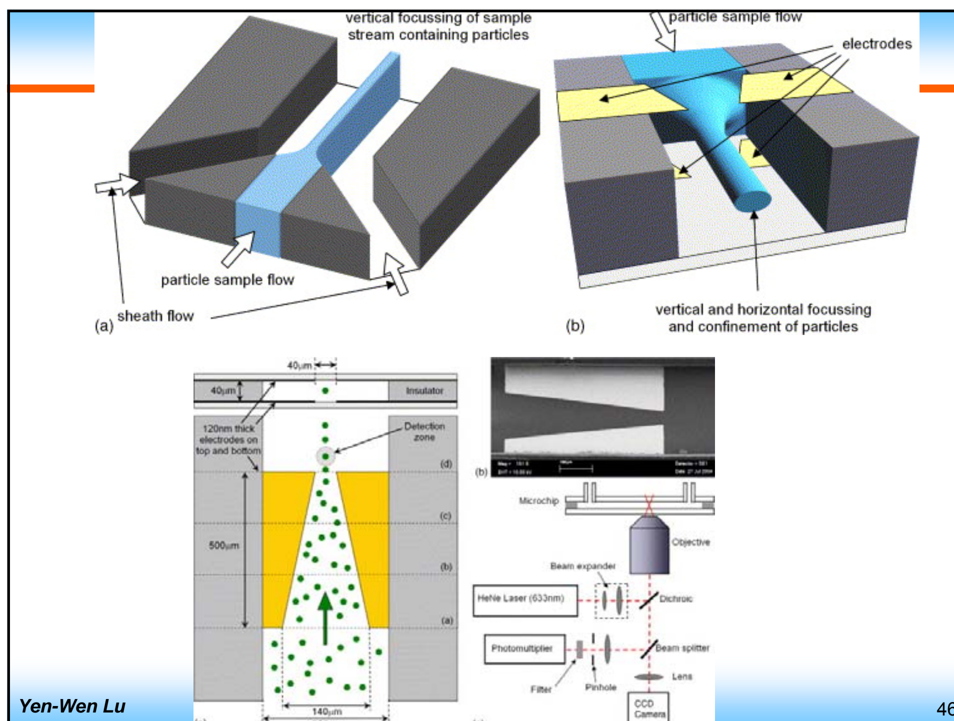
(a) (b) Microbeads in the channel
(a) w/o DEP (b) nDEP
(c) (d) Mouse neural stem cells flowing in the channel are switched

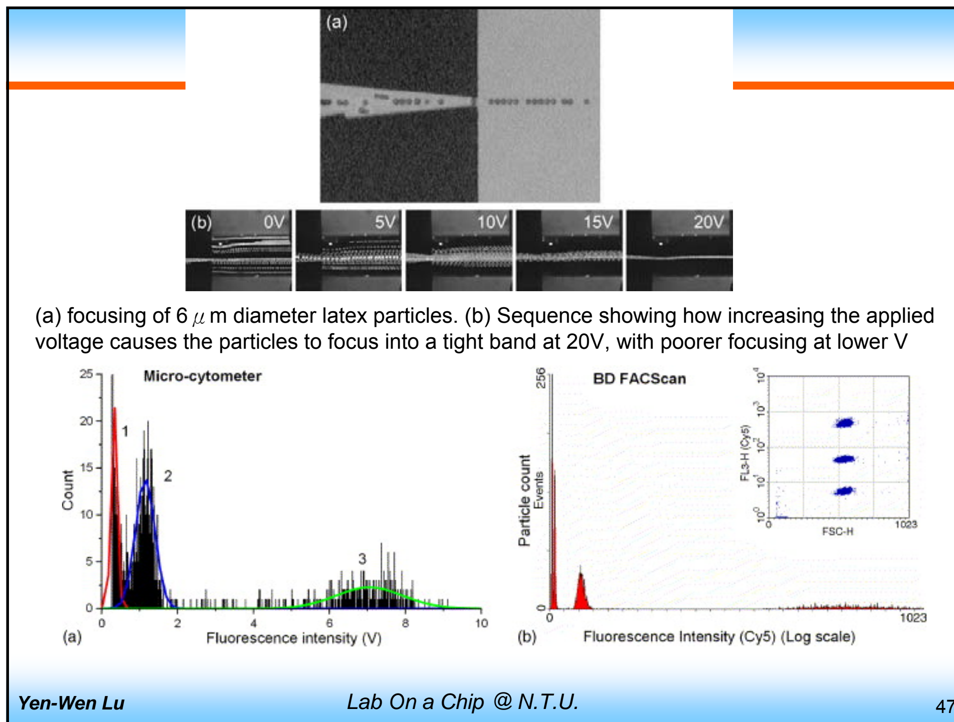
DEP switching of the microbeads to five different outlets The frequency applied on all the electrodes is 10 MHz and the voltage applied on the left and right electrodes are A(0,10), B(4,10), C(10,10), D(10,4), E(10,0). The microbeads are circled to clearly show the switching.

High throughput particle analysis: Combining DEP particle focusing w/ confocal optical detection

A micro flow cytometer has been fabricated that detects and counts fluorescent particles flowing through a microchannel at high speed based upon their fluorescence emission intensity. Dielectrophoresis is used to continuously focus particles within the flowing fluid stream into the centre of the device, which is 40 μm high and 250 μm wide. The method ensures that all the particles pass through an interrogation region approximately 5 μm in diameter, which is created by focusing a beam of light into a spot. The functioning of the device was demonstrated by detecting and counting fluorescent latex particles at a rate of up to 250 particles/s. A mixture of three different populations of latex particle was used, each sub-population with a distinct level of fluorescent intensity. The device was evaluated by comparison with a conventional fluorescent activated cell sorter (FACS) and numerical simulation demonstrated that for 6 μm beads, and for this design of chip the theoretical throughput is of the order of 1000 particles/s (corresponding to a particle velocity of 10 mm s^{-1}).

Biosensors and Bioelectronics 21 (2006) 1621–1630




(a)
(b) 0V
5V
10V
15V
20V
(a) focusing of 6 μm diameter latex particles. (b) Sequence showing how increasing the applied voltage causes the particles to focus into a tight band at 20V, with poorer focusing at lower V
Micro-cytometer
Count
Fluorescence intensity (V)
BD FACScan
Particle count
Events
Fluorescence Intensity (Cy5) (Log scale)
FSC-H
FL3-H (Cy5)
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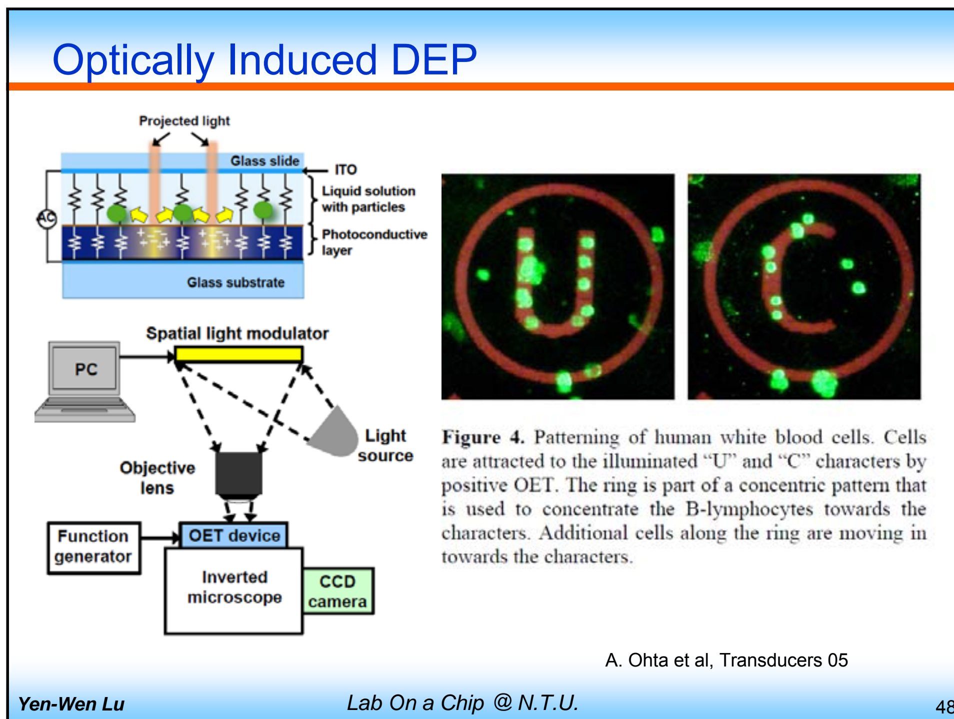
Optically Induced DEP
Projected light
Glass slide
ITO
Liquid solution with particles
Photoconductive layer
Glass substrate
AC
Spatial light modulator
PC
Light source
Objective lens
Function generator
OET device
Inverted microscope
CCD camera
Figure 4. Patterning of human white blood cells. Cells are attracted to the illuminated "U" and "C" characters by positive OET. The ring is part of a concentric pattern that is used to concentrate the B-lymphocytes towards the characters. Additional cells along the ring are moving in towards the characters.
A. Ohta et al, Transducers 05
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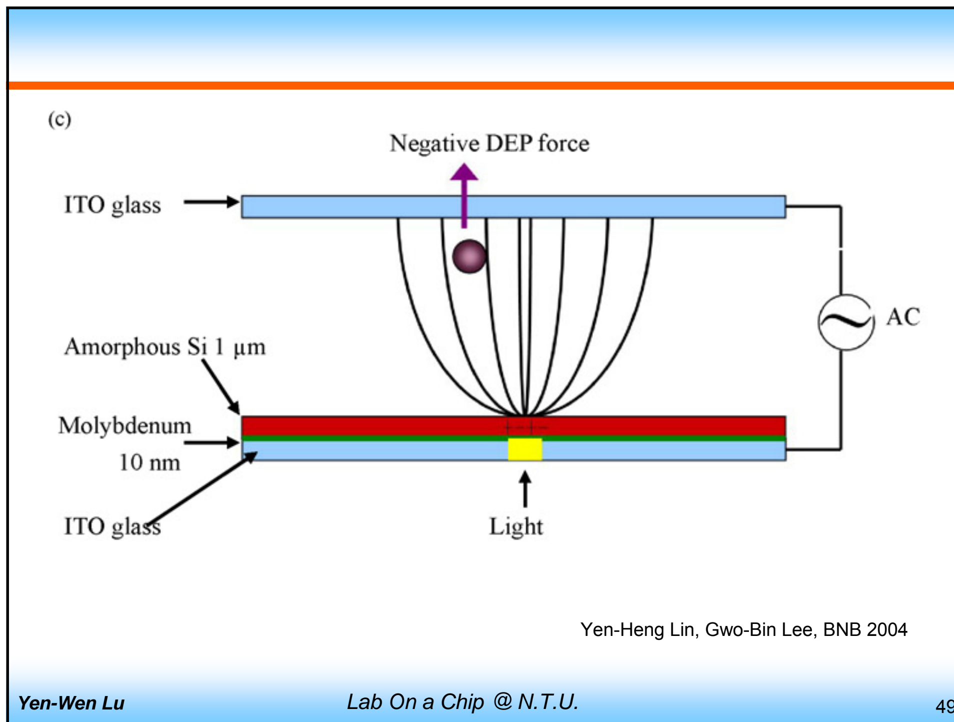


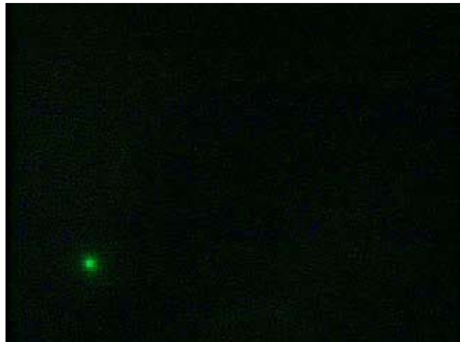
Optically Induced DEP

ITO glass
Projected image
a.c.
Glass substrate
Nitride
Undoped a-Si:H
n+ a-Si:H
ITO
10x
Light-emitting diode
DMD microdisplay

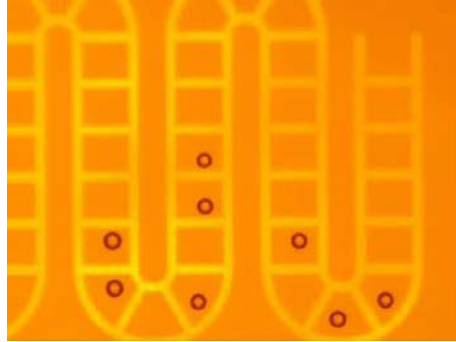
Liquid that contains microscopic particles is sandwiched between the top ITO glass and the bottom photosensitive surface consisting of ITO-coated glass topped with multiple featureless layers: 50 nm of heavily doped a-Si:H and 20 nm of silicon nitride. The top and bottom surfaces are biased with an AC electric signal. The illumination source is an light-emitting diode operating at a wavelength of 625 nm. The optical images shown on the digital micromirror display (DMD) are focused onto the photosensitive surface and create the non-uniform electric field for DEP manipulation

Massively parallel manipulation of single cells and microparticles using optical images by P.Y. Chiou et al. Nature (2005)

Optically Induced DEP (Demo)

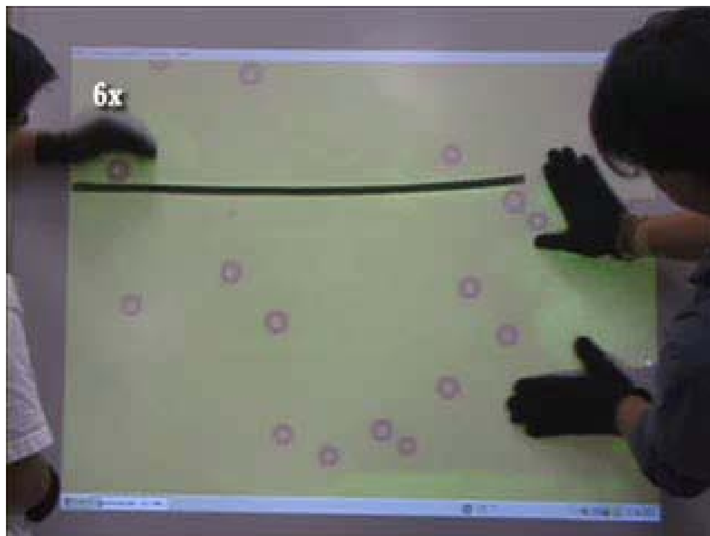


Trapping and releasing of live *E. Colis* .



Conveyer transporting 20 um polystyrene beads

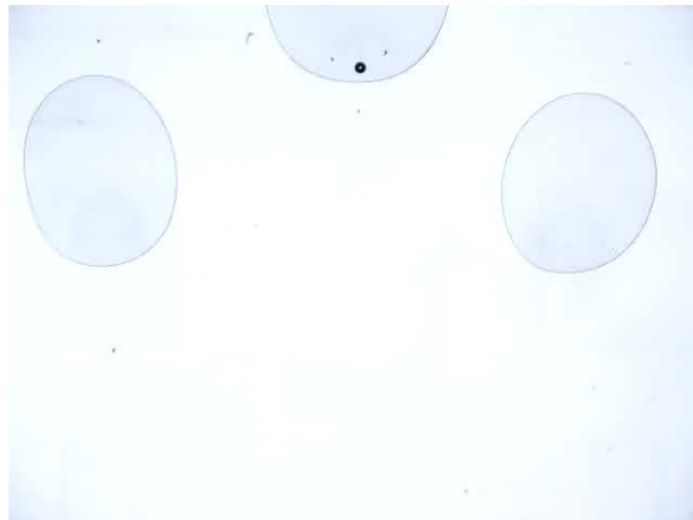
Optically Induced DEP (Demo)



Real-time interactive OET using image feedback control

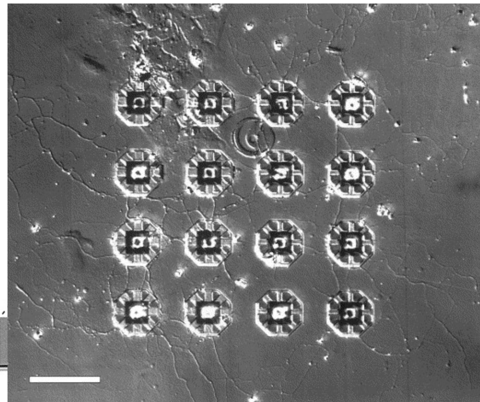
Take-Home Messages

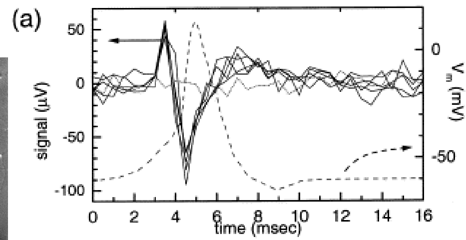
- Cytometer:
  - Light scattered by a laser or arc lamp
  - Specific fluorescence detection
  - Hydrodynamically focused stream of particles
  - Electrostatic particle separation for sorting
  - Multivariate data analysis capability
- Methods to manipulate particles/cells
  - Hydrohynamic focusing
  - DEP

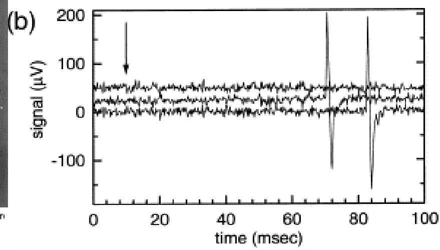


Cell Trapping

- Immobilize cells to be observed or assayed for a long time.







MP. Maher et al. 1999

PDMS Microwells

Hydrohynamic Traps