# The $1000 Genome: Sequencing DNA One Drop at a Time

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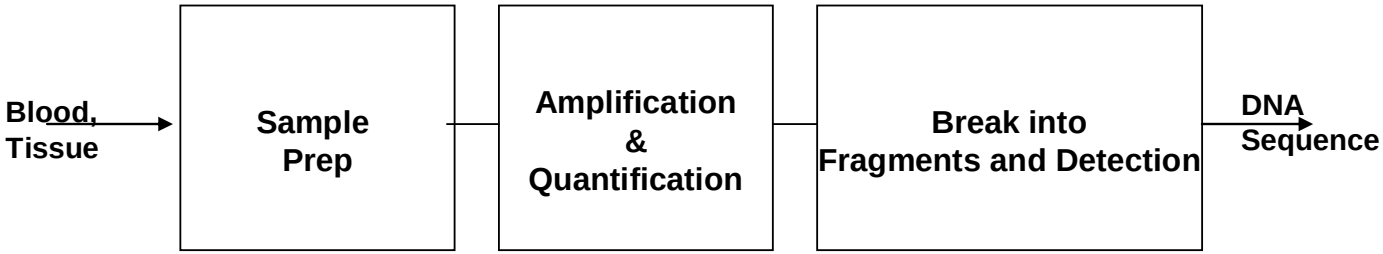
# DNA Sequencing

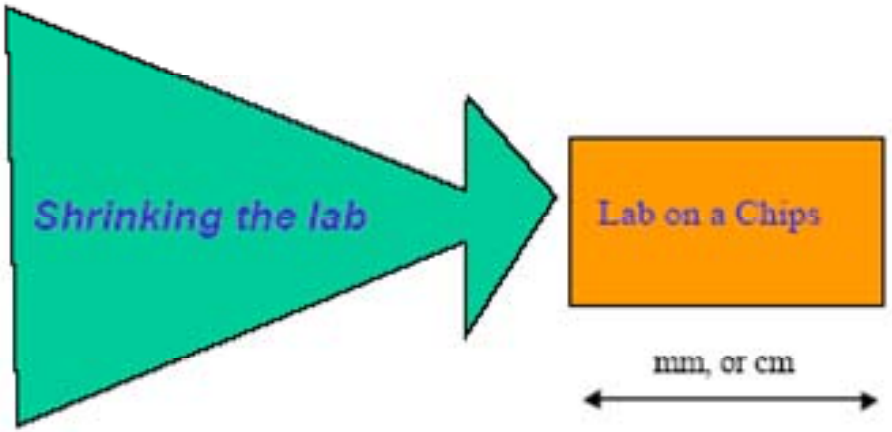
- What is it?
  - Process of determining the sequence of nucleotides making up the length of DNA
- How is it typically done?




- What equipment is required?

# Promise of Microfluidics

Applications : Biotechnology (eg: high throughput screening , Diagnostics...)

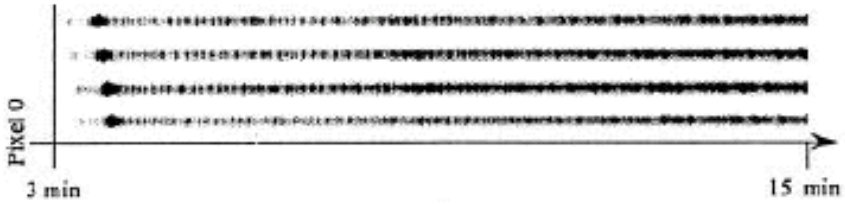



Can a lab-on-a-chip be as versatile as the macro lab it replaces?

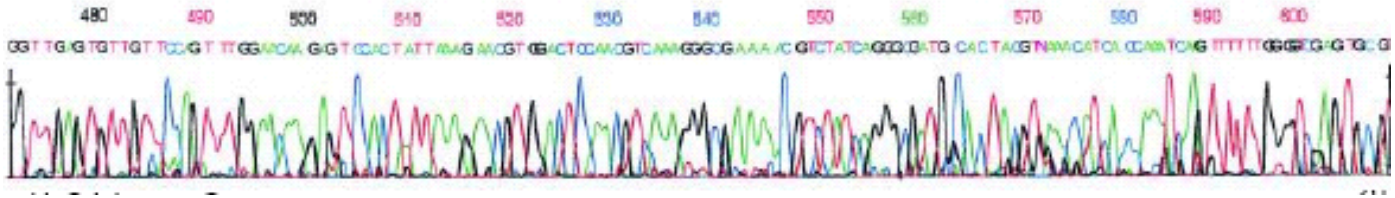
# Chip-Based Sequencing

## Sequencing On-Chip

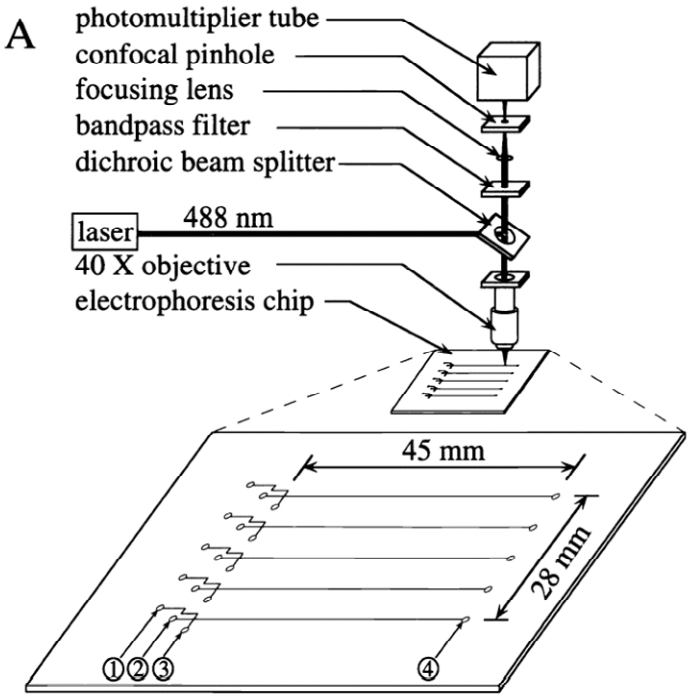
- DNA sequencing on a microchip
  - First demonstrated in 1995 by Mathies group, UCB:
    150 bases in 540 s with 97% accuracy
  - In 2002, 96-channel plate demonstrated:
    430 bases read in parallel at average rate of 1.7 kb/min
    with >99% accuracy

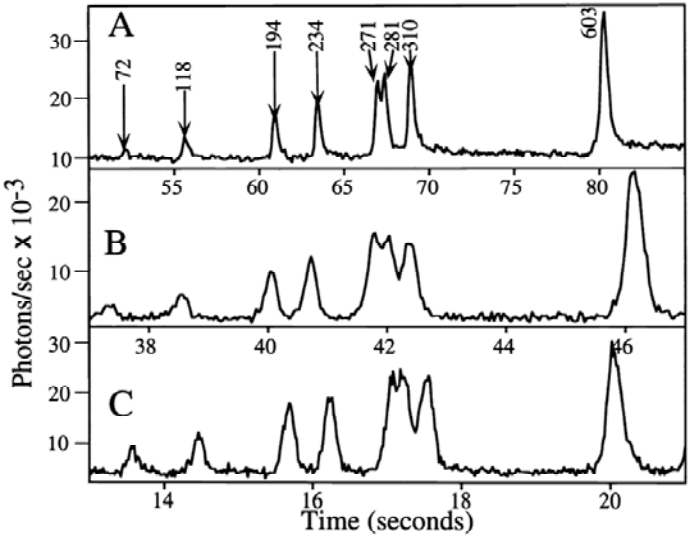



*Mathies group*



# Sequencing Chip-1995

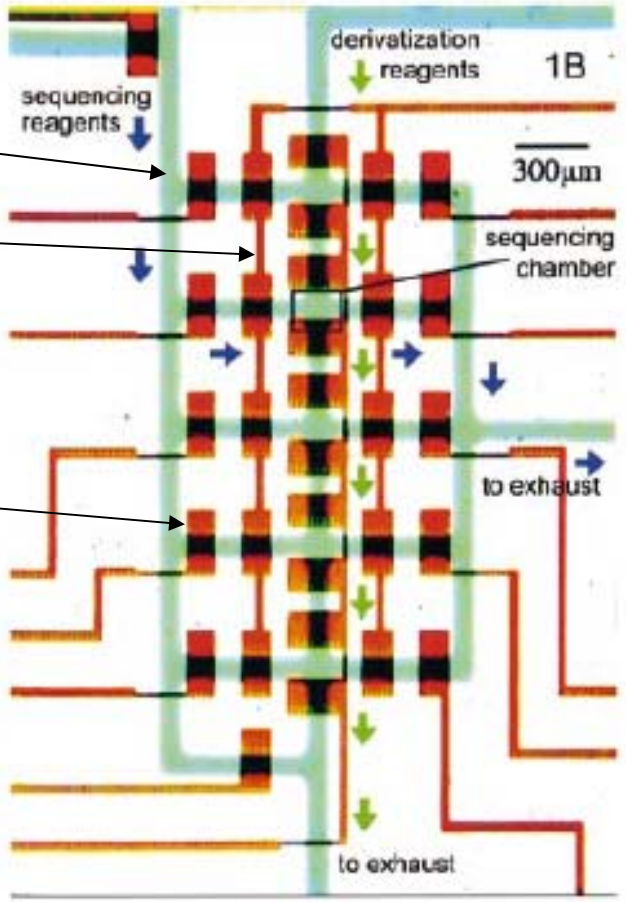


# Sequencing by Synthesis -2004

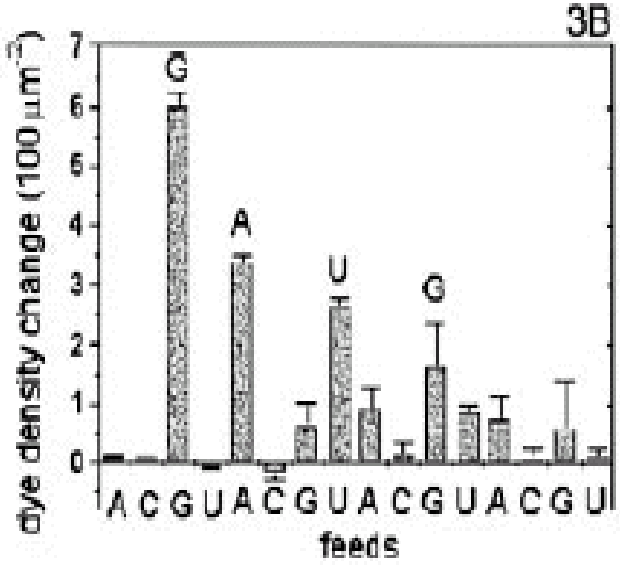
## (Kartalov and Quake)

- Green-flow channels
- Red-control layer
- 5 sequencing chambers
- Valves formed where wide red crosses green



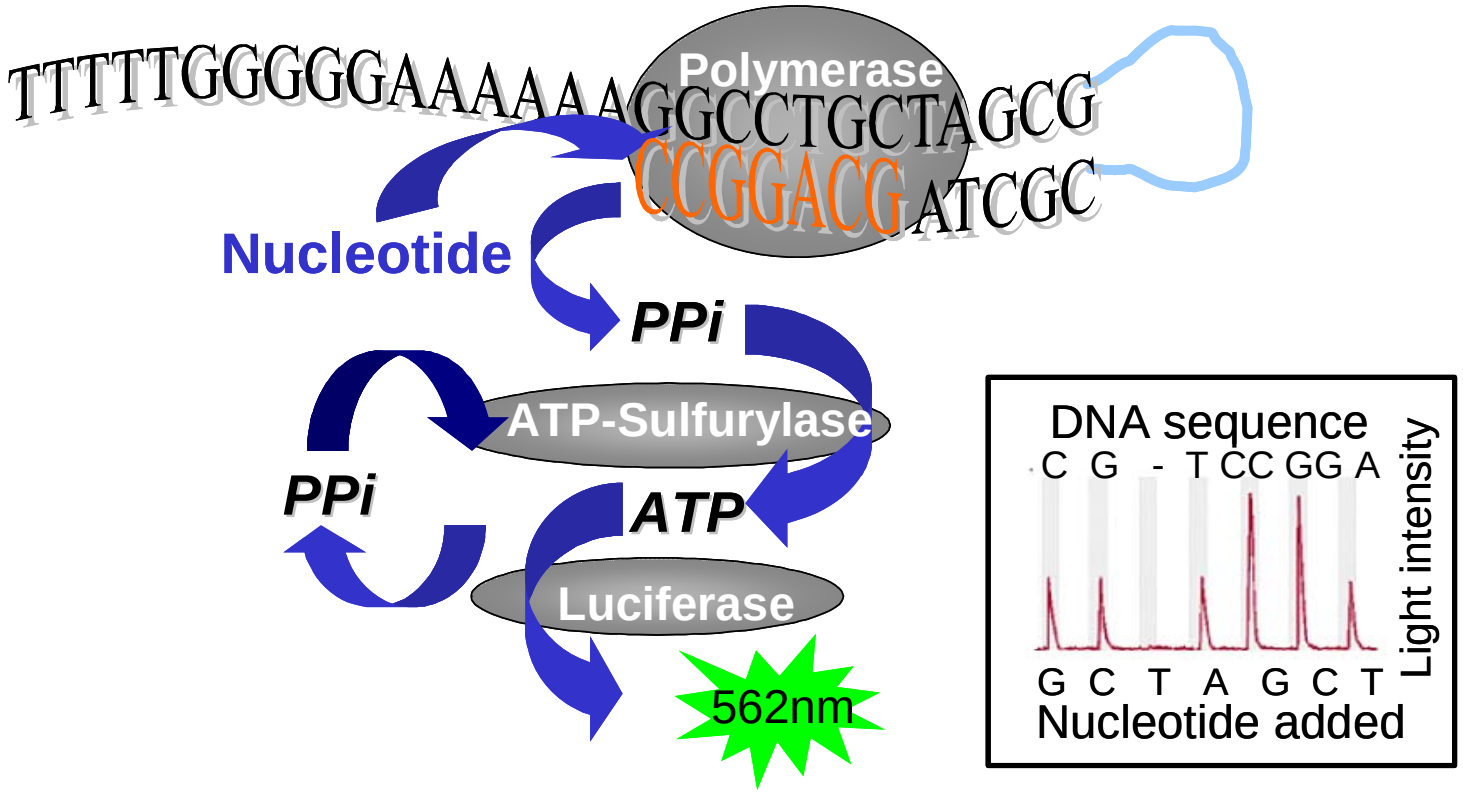
# Sequencing by Synthesis

- Signal/noise ratio degrades – limits read length to 4bp
  - non-specific binding tagged nucleotides to channel walls
  - Reduced amount of DNA available with each incorporation step



# NHGRI Genome Technology Program

- The Genome Technology program supports research to develop new methods, technologies and instruments that enable rapid, low-cost determination of DNA sequence, SNP genotyping (Genetic Variation Program) and functional genomics (broadly defined) experiments (Functional Analysis Program). Priorities include the refinement of current technologies to increase efficiency and decrease cost while maintaining or improving data quality, and the development of completely novel approaches to achieve orders-of-magnitude improvement. Integration of process steps is key to achieving these goals.




$$DNA_N + dNTP \xrightarrow{\text{Polymerase}} DNA_{N+1} + PPi$$

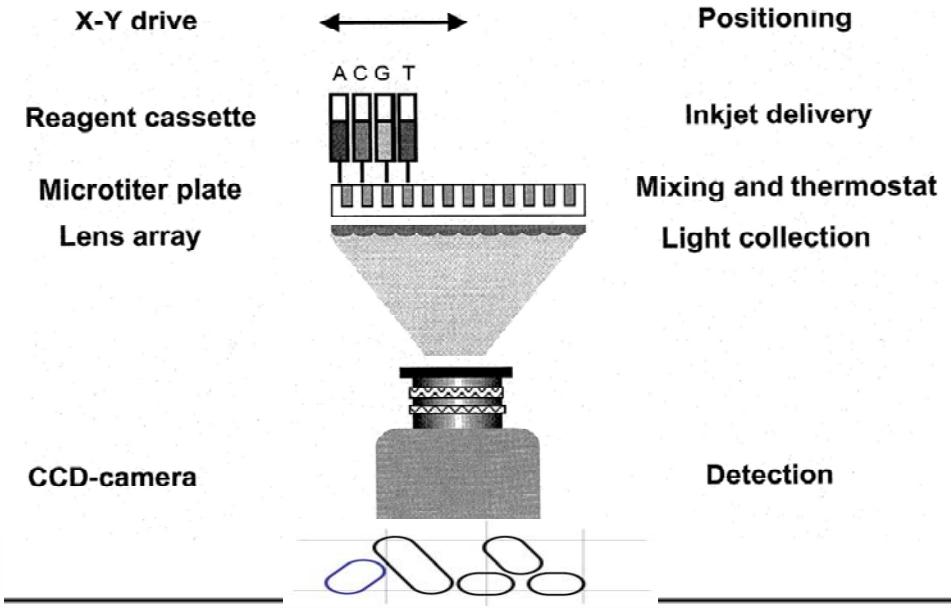
**Coupled Reactions**

$$PPi + APS \xrightarrow{\text{Sulfulylase}} ATP$$

$$ATP + O_2 + \text{Luciferin} \xrightarrow{\text{Luciferase}} AMP + PPi + CO_2 + \text{Oxyluciferase} + \text{Light}$$

# Liquid Phase Pyrosequencing

- Primed DNA template and 4 enzymes are placed in a microtiter plate well
- 4 different nucleotides are added stepwise
- Incorporation is followed using enzymes ATP sulfurylase and luciferase
- Requires addition of nucleotide-degrading enzyme between steps

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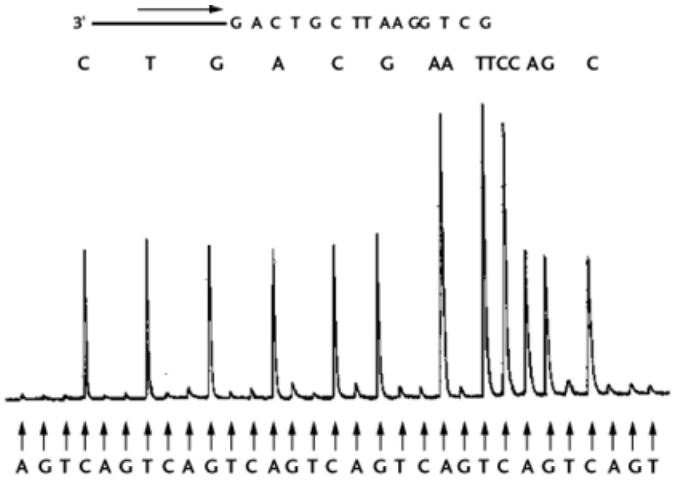
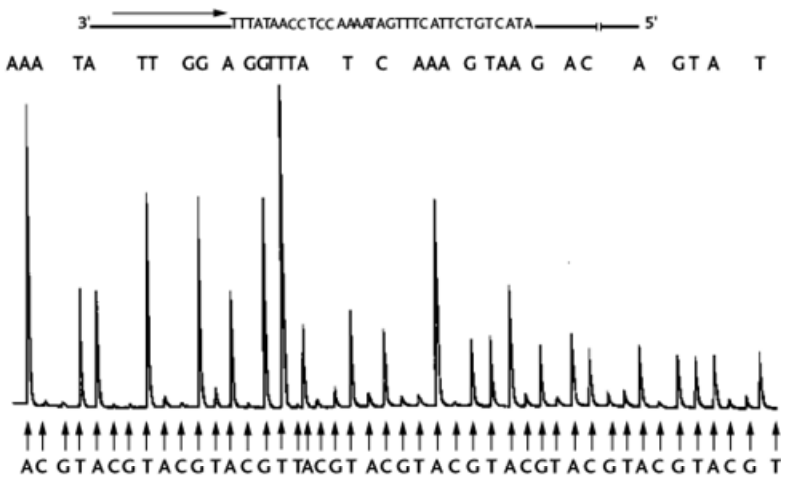
# Pyrosequencing Results

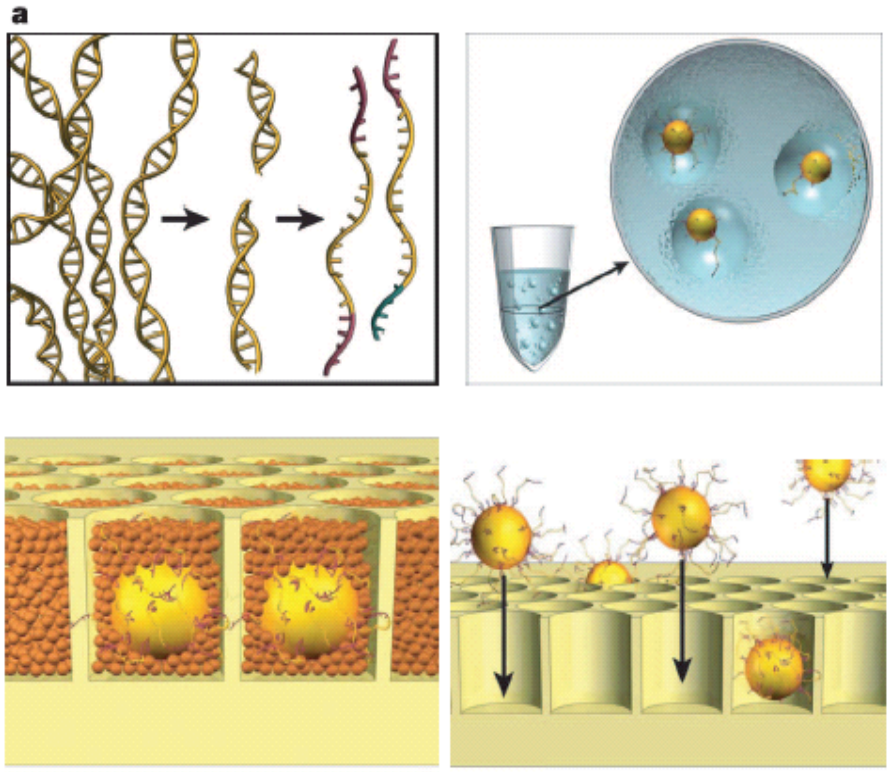
35 long base DNA

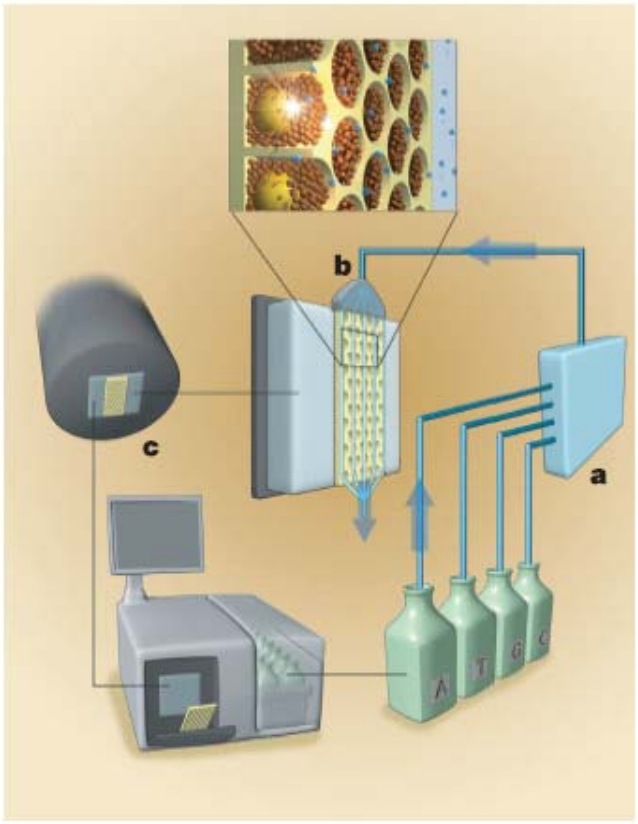



130 long base DNA

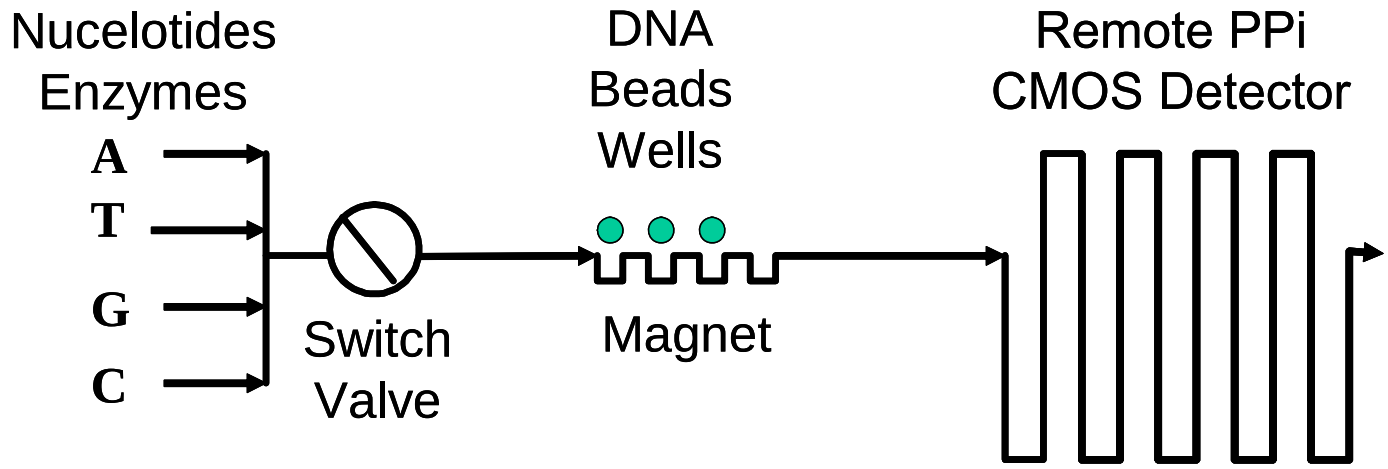


# 454 Life Sciences



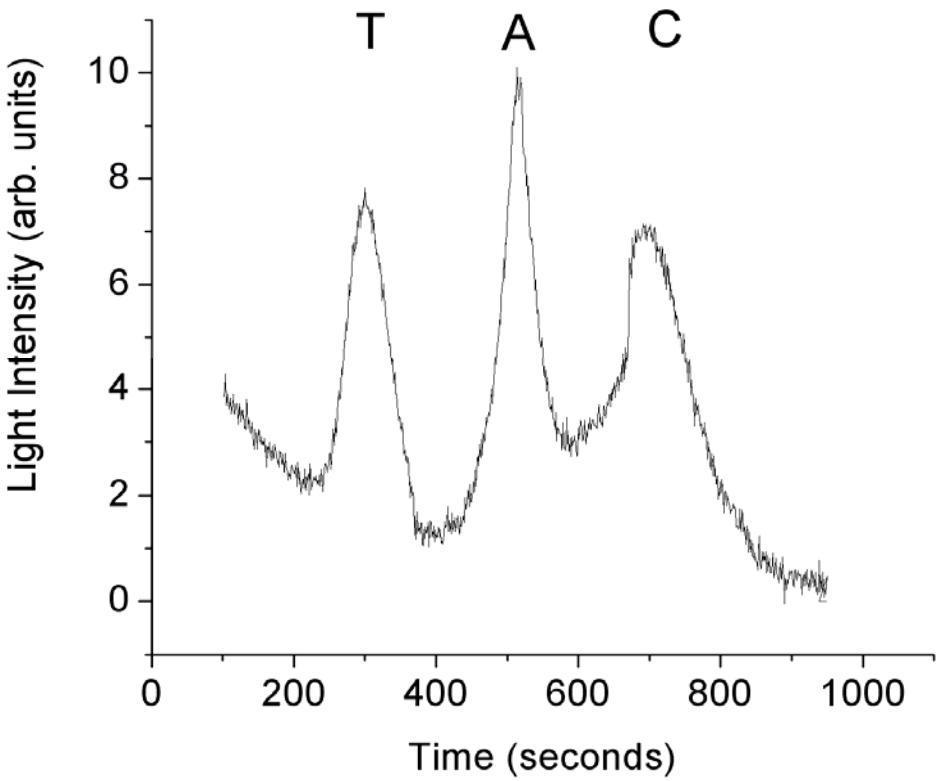



# On Chip Pyrosequencing




- Continuous flow of fresh reagents past anchored DNA
- Regenerative PPi – Photon chemiluminescent reaction
- Optimal downstream detection volume
- System can be used for fundamental studies of flow/reaction sequencing kinetics and optimization

# Initial CF Sequencing Results



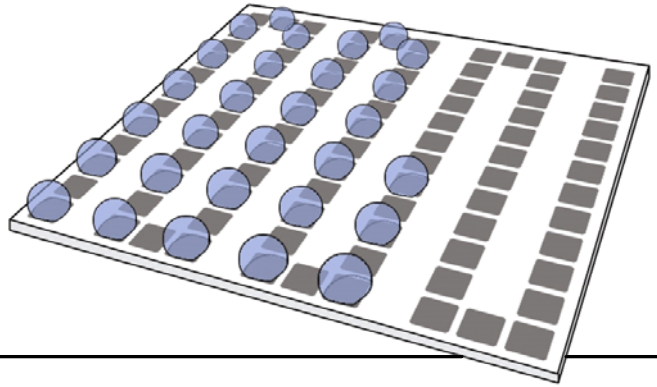
# Advantages

- Separate the synthesis reaction from the detection site in time and space
- Optimized synthesis reactions possible by continuous flow of fresh reagents
- Elimination of by-product build-up at synthesis site
- Potential for sequencing at the natural rate of DNA synthesis
- Scale-up path from microfluidic channels to single droplets of reagents transported by electrowetting

# Digital Microfluidics

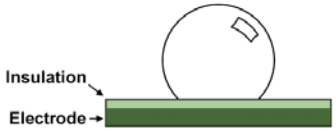
## Features

- Droplet-based microfluidic devices
  - Droplets are moved in “virtual channels” defined by electrodes
  - Programmable electrodes in an array directly control discrete droplet operations – dispense, transport, mix, split, incubate – to perform any liquid-based test

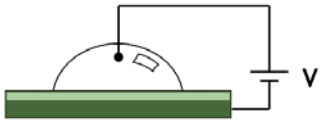


## How It Works

Voltage Off:




Voltage On:




- Electrowetting
  - Modulation of solid-liquid interfacial tension by the application of an electric field
- Works with or without a top plate
  - Newly developed coplanar electrowetting method

# Technology Advantages for Sequencing

## Digital Microfluidics

- Very accurate droplet volumes
- Droplet-based digital microfluidics is functionally more similar to bench protocols
  - Assays more easily adapted
- Programmable, software-driven electronic control
  - No moving parts, tubes, pumps or valves
- More efficient use of samples and reagents
  - No liquid is wasted priming channels
- Extremely energy efficient
  - Suitable for low power and portable applications
- Development cycles are short, and assays can be tuned with software changes
- Low cost, production-ready lab-on-a-chip on printed circuit board substrate

## Other Microfluidic Technologies

- Pump fluids through channels
- Must adapt assays to channel-based format
- Complex or multiplexed assays become a plumber's nightmare
- Off-chip pumps and valves mean large, expensive equipment and low reliability
- Expensive, time consuming, up-front investments required for most chip developments
- Designs are fixed in the development process

# Digital Microfluidic Toolkit

**Implementing numerous applications on a elemental set of components:**

Reservoirs ➡ droplets
Dispensers ➡ electrode sets
Pumps ➡ electrode sets
Valves ➡ electrode sets
Reaction vessels ➡ droplets
Mixers ➡ electrode sets
Collection ➡ scanning droplet

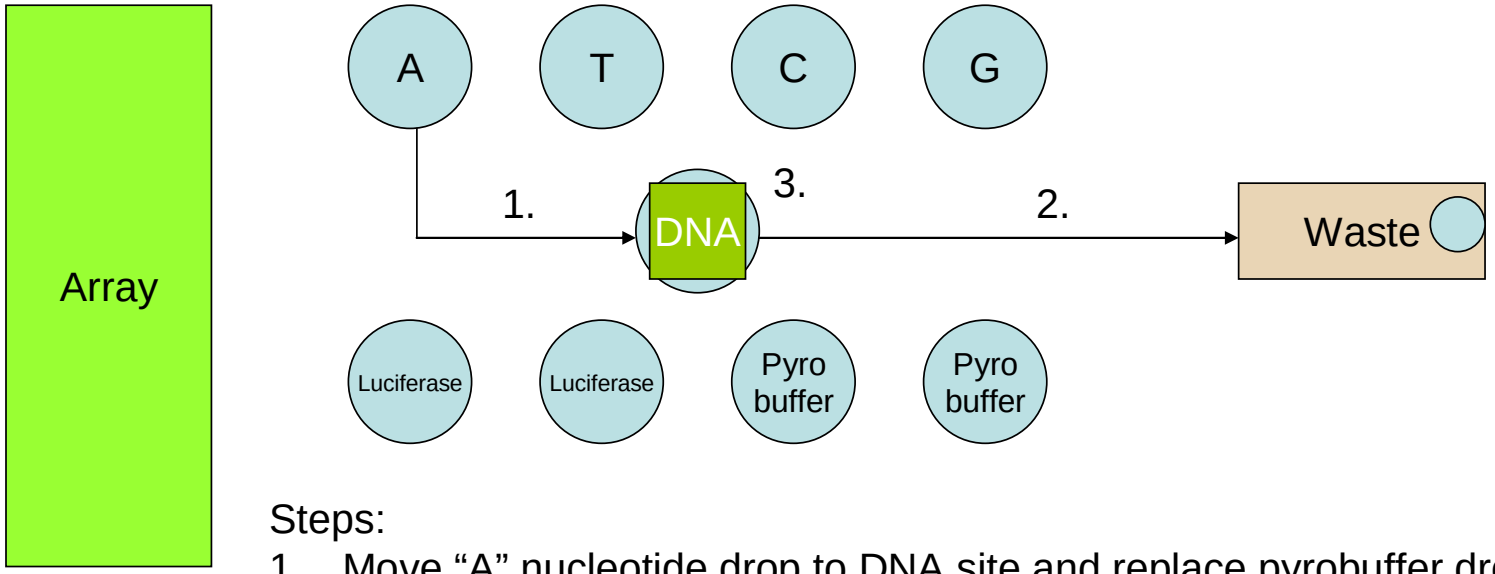
# Benefits of Reconfiguration in Pyrosequencing

- Enzyme contamination of chip surfaces may occur
  - Route wash droplets over transport buses for reuse
- Feedback-controlled nucleotide addition for sequencing through homopolymer regions of DNA
- Look-ahead sequencing and voting schemes possible for reliable and high throughput sequencing
- Other benefits of digital microfluidic platform:
  - Continuous droplet dispensing
  - Scalable

# Justification for Droplets

- The use of droplet-based digital microfluidic technology based on electrowetting will allow the decoupling of synthesis and detection so that each can be individually optimized
- The scalability of this technology will allow the reduction of sequencing costs through decreased reagent volume and decreased instrument cost
- Massively parallel assays possible

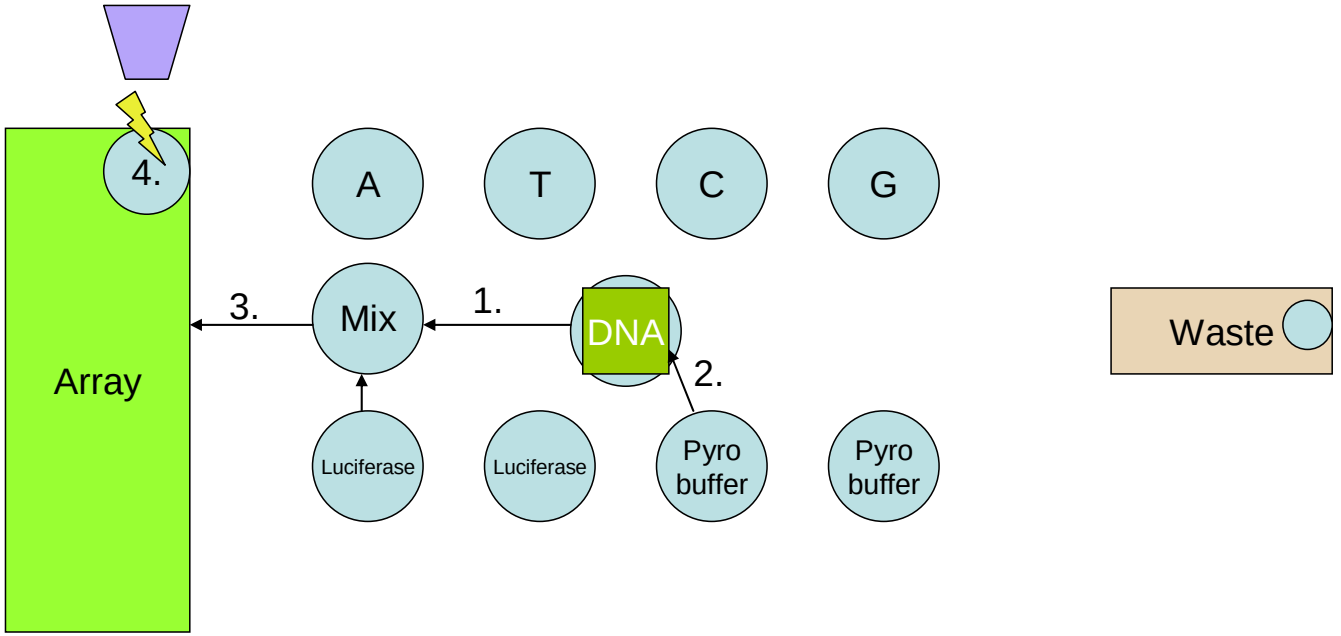
# Droplet-Based Sequencing




Steps:

1. Move “A” nucleotide drop to DNA site and replace pyrobuffer drop
2. Move pyrobuffer drop to waste
3. Incubate incorporation reaction

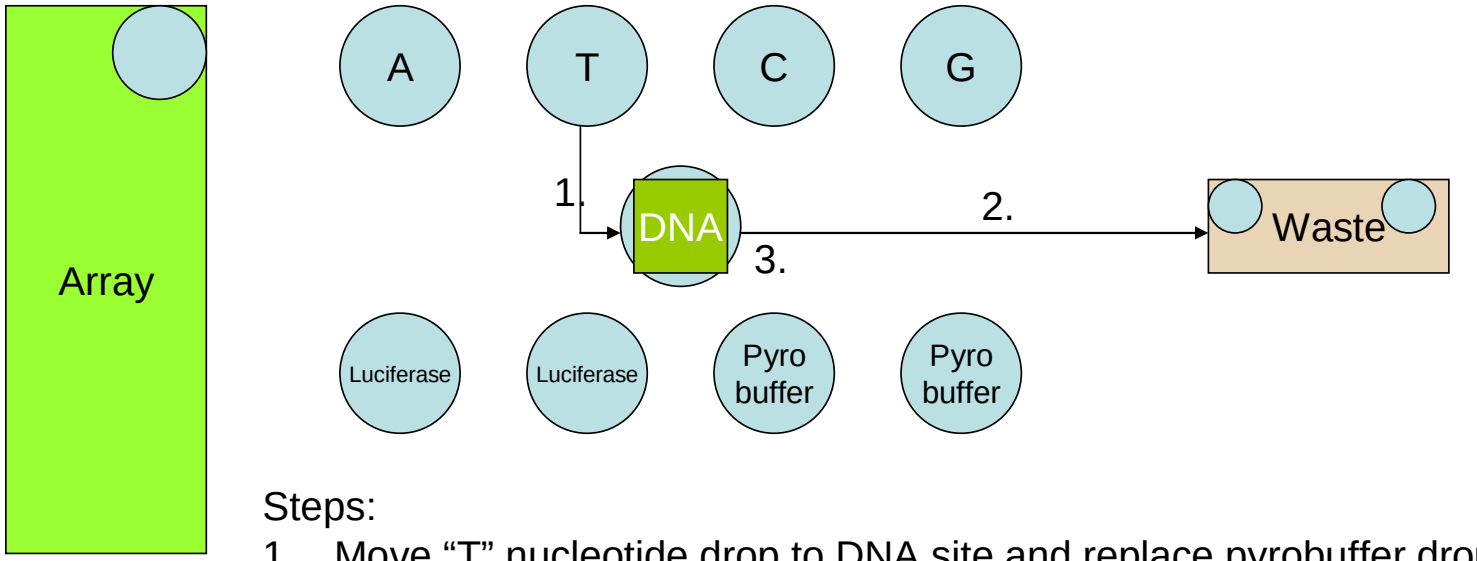
# Droplet-Based Sequencing




Steps:

1. Move incubated droplet and mix with luciferase
2. Move pyrobuffer drop and wash DNA (may be repeated)
3. Move combined droplet to array
4. Detect pyrophosphate generated light

# Droplet-Based Sequencing

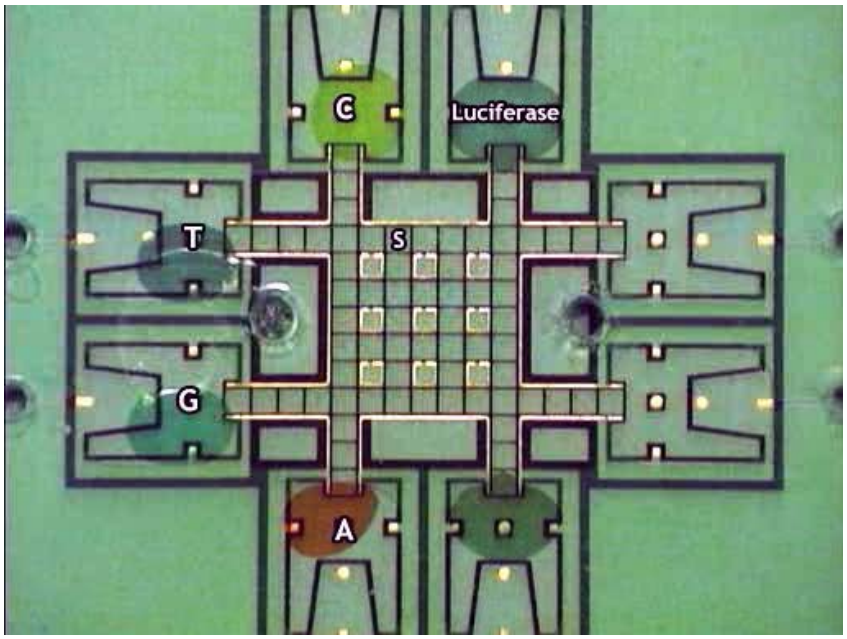



Steps:

1. Move “T” nucleotide drop to DNA site and replace pyrobuffer drop
2. Move pyrobuffer drop to waste
3. Incubate incorporation reaction

# Example Fluidic Protocol (2006)

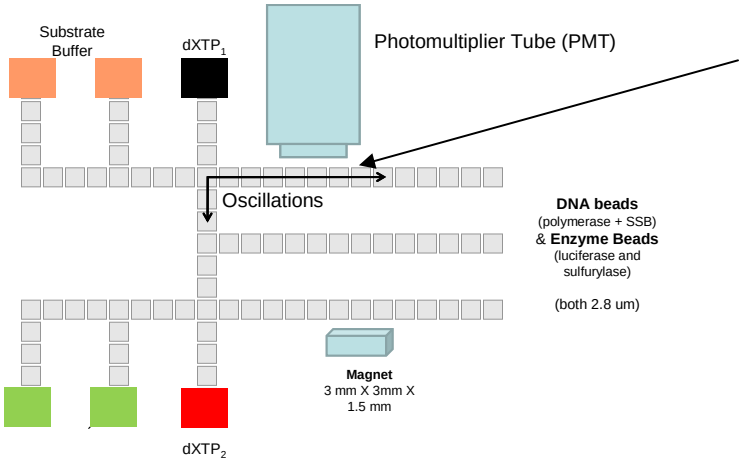
- Dispense droplets of each dNTP
- Transport droplets to synthesis reaction site and allow to react
- Transport droplets to storage area
- Mix each dNTP droplet with light producing droplet
- Transport combined droplets to detector site

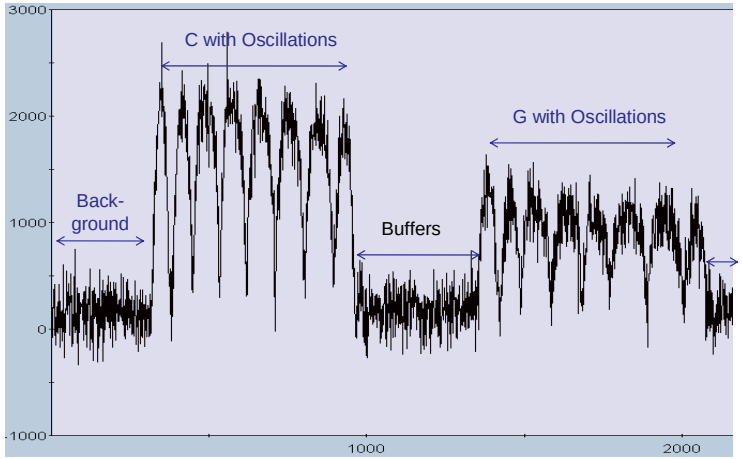



*Dyed liquids represent pyrosequencing reagents, droplet volumes are 50 – 100 nL.*

# On-Chip Pyrosequencing

- Pyrosequencing protocols run at Stanford on ALL platform having six reservoirs and three transport lanes.
- Magnet under bottom lane immobilizes DNA and enzyme beads
- After incorporation, reaction products transported to PMT

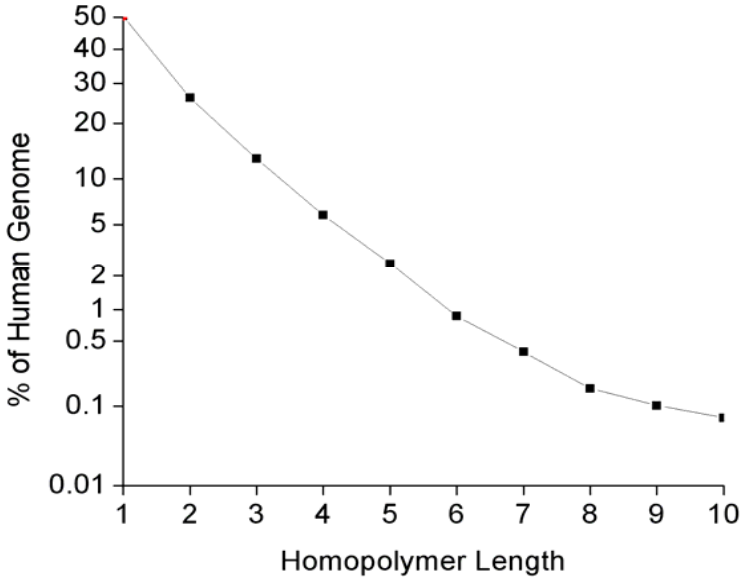


# Directions

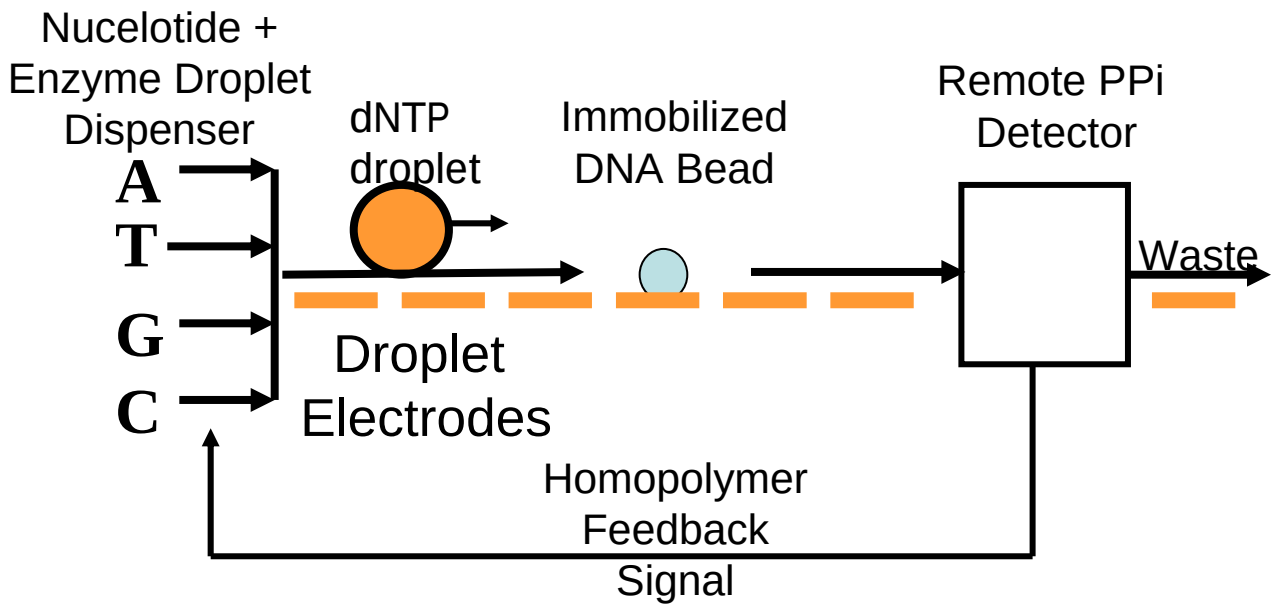
- Address issues in obtaining arbitrarily long reads
  - Continuous flow of fresh nucleotides and reagents to DNA
  - Advantages in separating synthesis and detection
    - Each can be individually optimized
    - Use feedback to add separately extra nucleotides at any DNA site where homopolymer regions are encountered
- Address complicated fluid handling to multiple parallel channels
  - Use discrete droplets under voltage control
  - Wash DNA between each step
- The scalability of this technology will allow the reduction of sequencing costs through decreased reagent volume and decreased instrument cost

# Human Homopolymer Runs



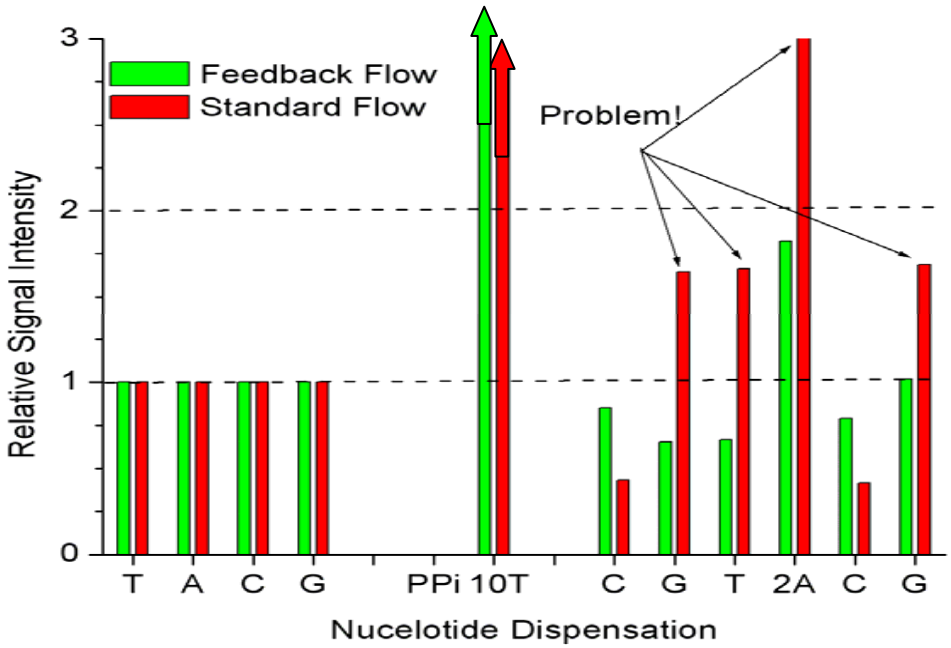

- **33% of 1000bp segments will contain 10-mers or longer (random dist).**
- **Shorter homopolymer runs are extremely common in human**
- **De-novo sequencing needs a strategy to sequence through homopolymers with high fidelity**
- **Feed-back delivery of extra nucelotide to homopolymer regions provides such a capability, potentially enabling very long read lengths**

# Feedback Controlled Nucelotide Addition For High Fidelity Homopolymer Sequencing




- Deliver dNTP droplet
- If excess light is detected (homopolymer), add more of same dNTP
- Continue adding same dNTP until full incorporation detected
- Else, deliver next nucleotide

# High Fidelity Homopolymer (10T) Sequencing in a Single Flowchannel

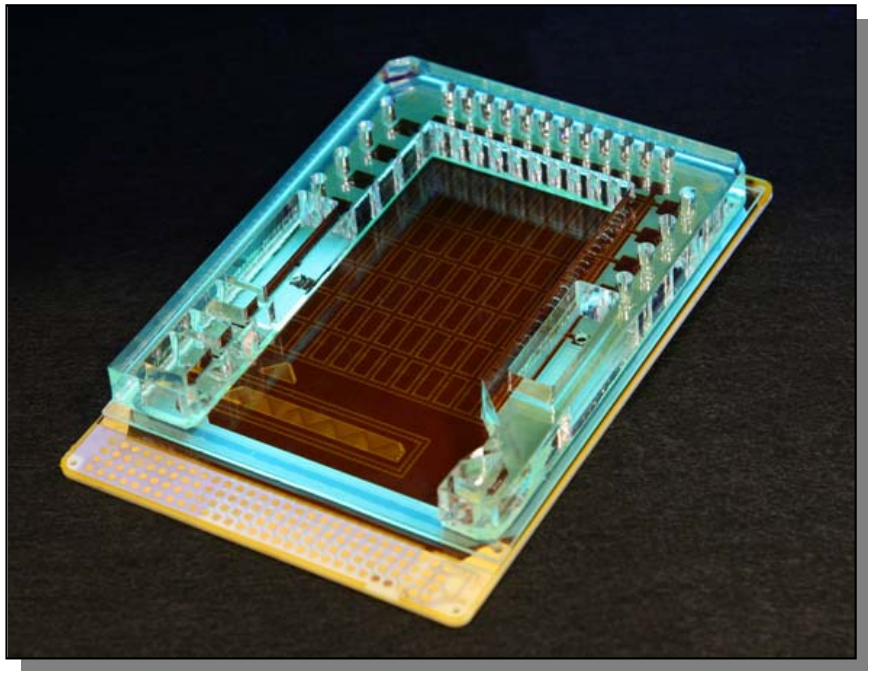



- Feedback controlled addition until 10T fully incorporated
- Without feedback, subsequent dNTP misincorporation and evidence of incomplete T incorporation.

# Platform Compatibility Requirements

- Manipulation of pyrosequencing reagents on-chip
  - Requires liquids compatible with electrowetting platform
  - No cross-contamination
  - No contamination from platform
- Use DNA attached to magnetic beads.
  - bead-washing requires 100% bead retention
  - No loss of DNA
- Assay development requires fully automated operation
- Automatic generation of thousands of droplets

# World to Chip Interface



- Well-plate interface
  - Easy and familiar loading
  - 384-well spacing
  - Inputs from microliters to milliliters
- Wash/waste reservoirs support 48+ tests
  - Load and go

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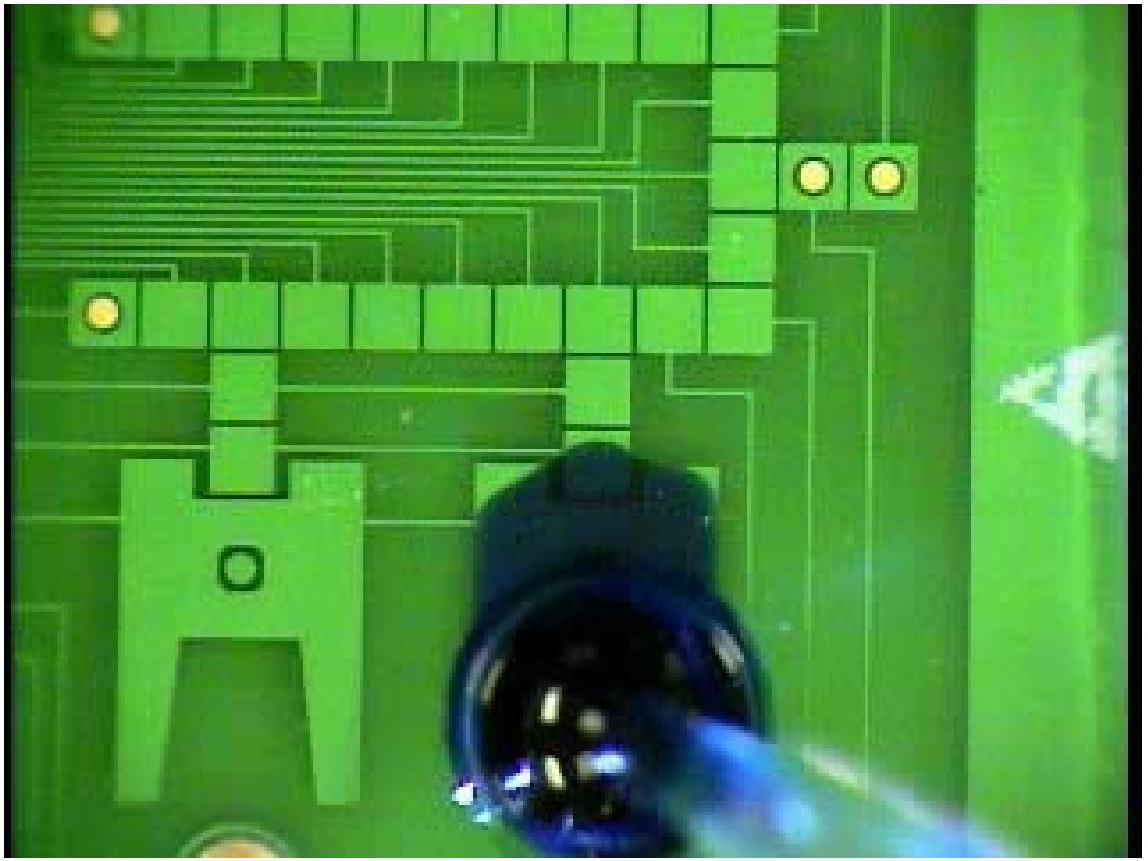
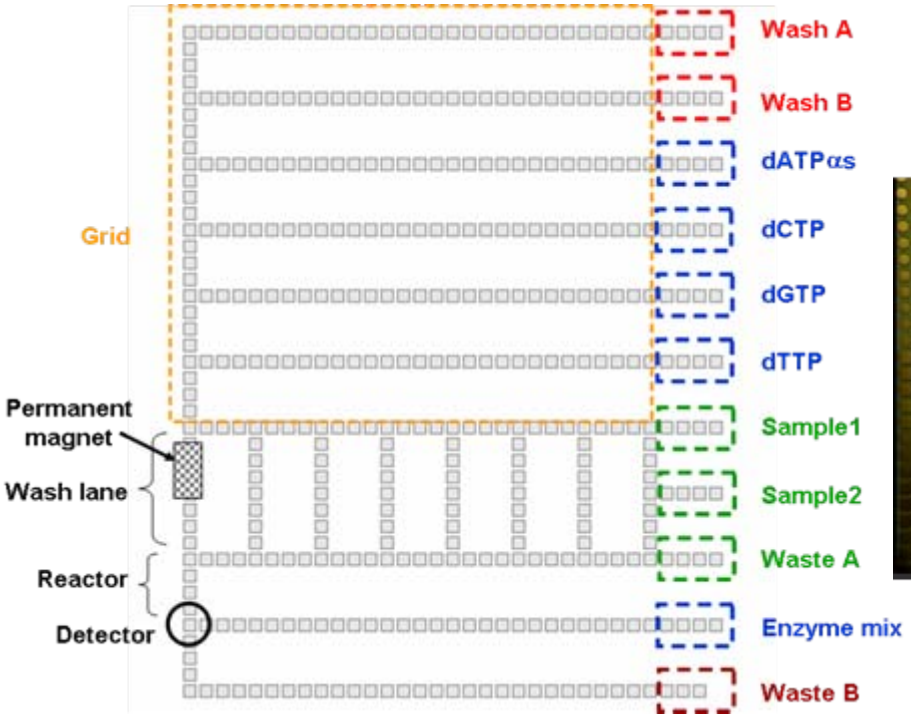
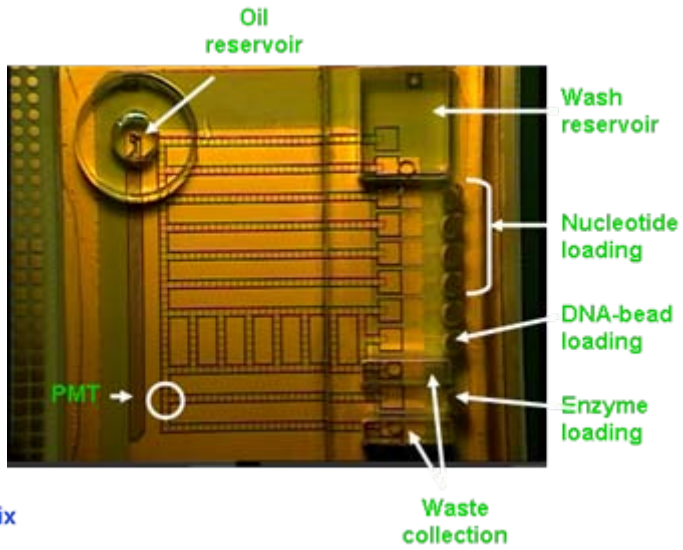
# High Speed Continuous Droplet Dispensing

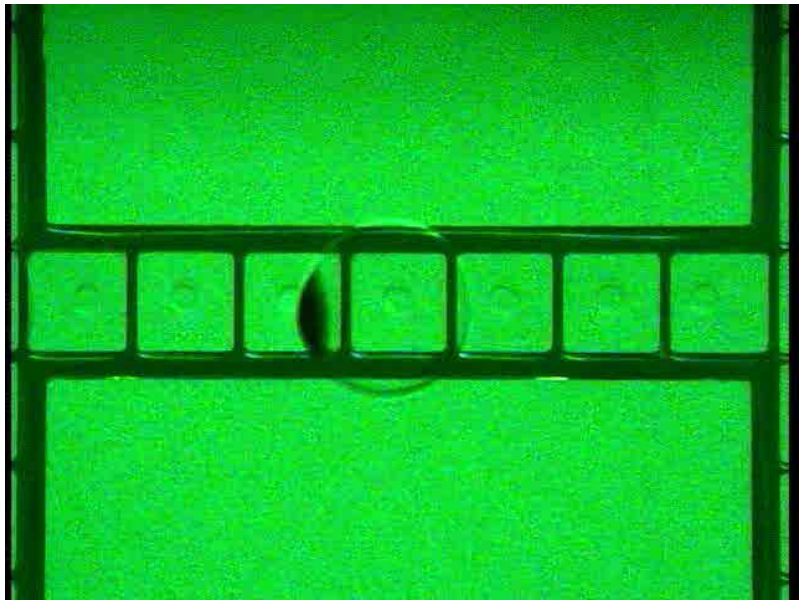


# Chip Architecture



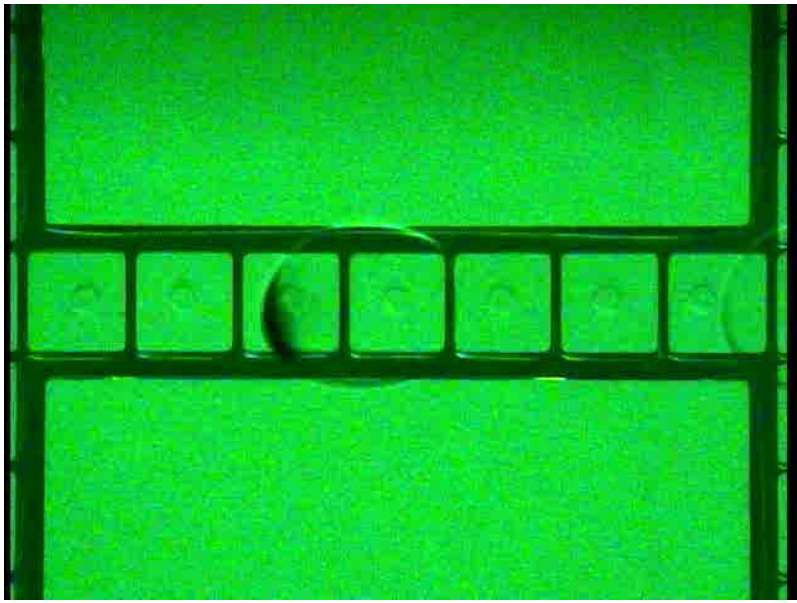



# Magnetic Bead Washing



One complete wash cycle

1X speed video of first wash cycle

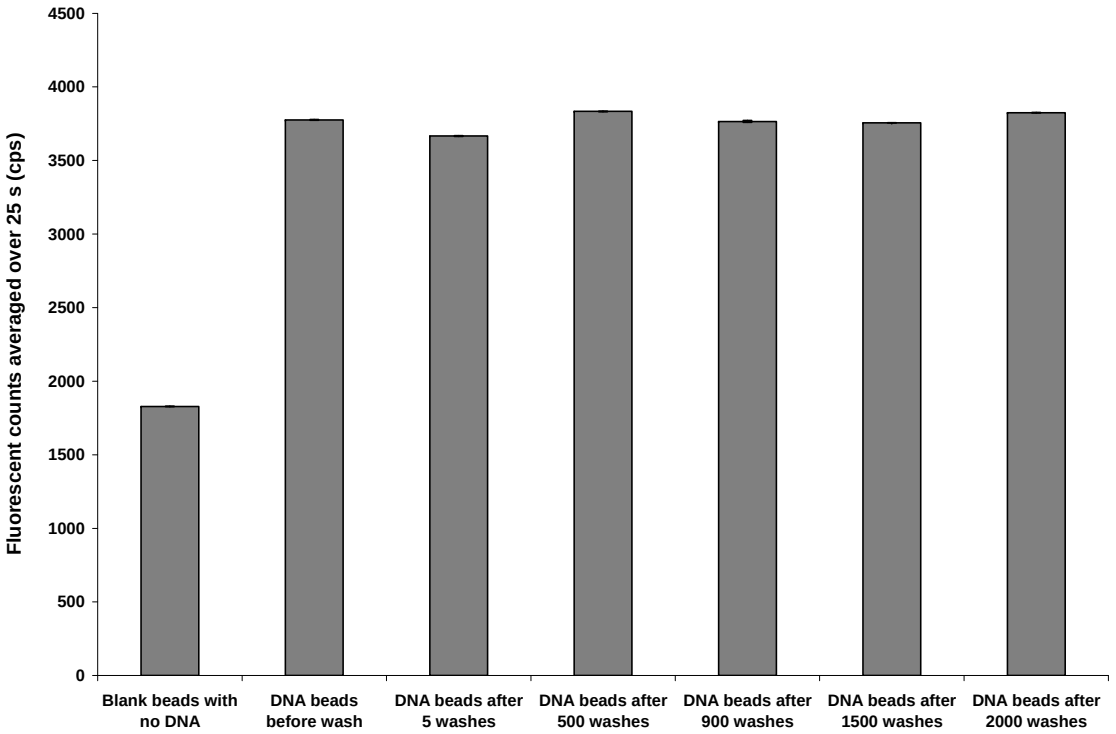


800 wash cycles (condensed to 26 s)

Still images taken after completion of each wash cycle are played at 30 fps

# Bead Washing Results

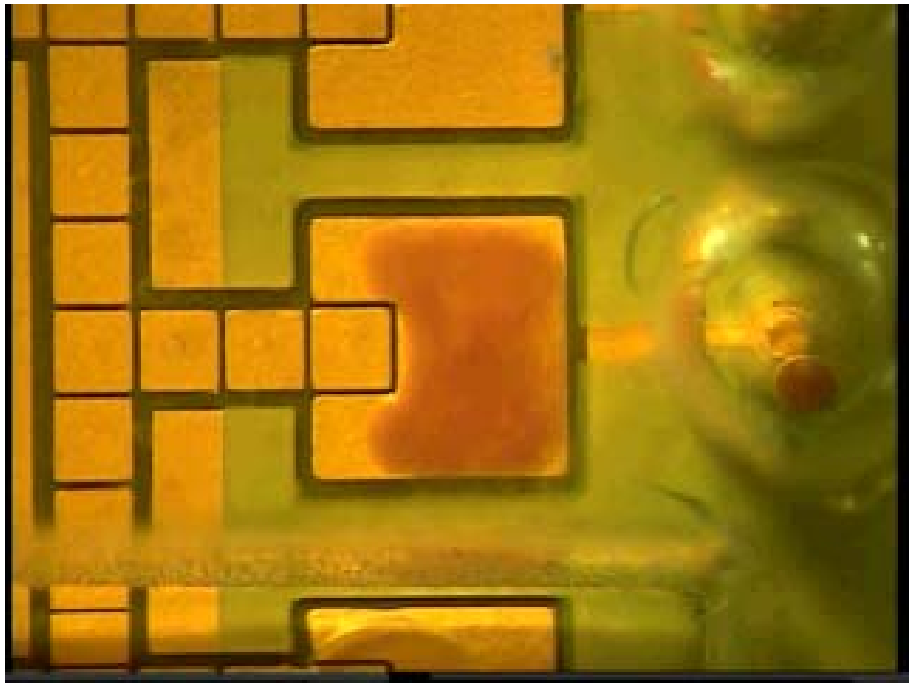
- Fluorescence of the FAM-labeled primer/DNA attached to beads monitored with washes



# On-Chip Pyrosequencing Protocol

**Steps involved**

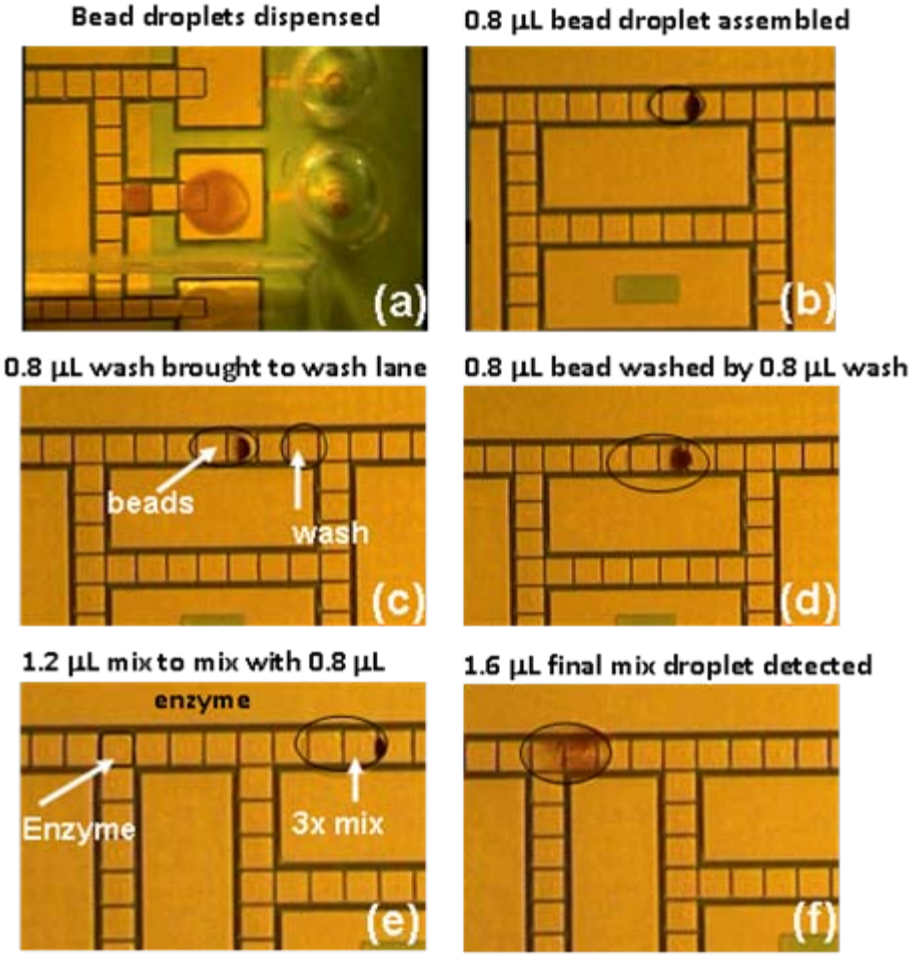
- Dispense beads (sample)
- Wash beads
- Add dNTP mix to beads
- Add enzyme mix to beads
- Mix & Detect



# Experimental Methods

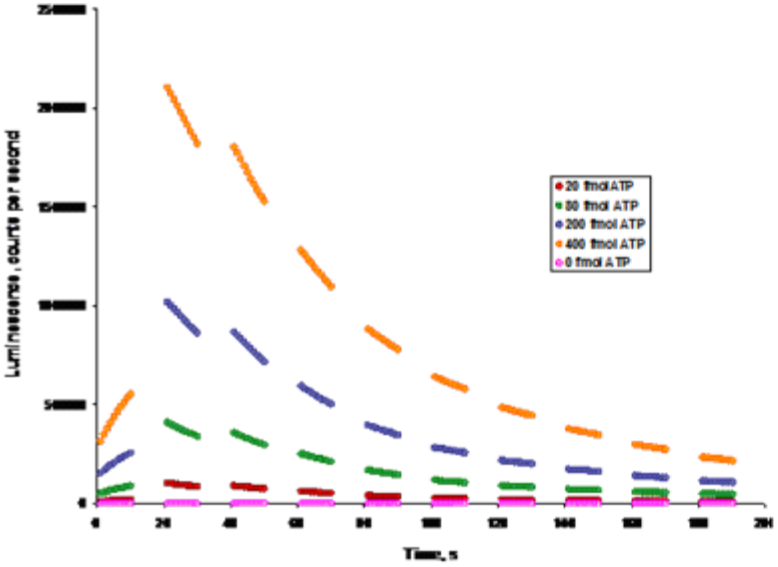
- *DNA templates: Candida albicans* genomic DNA (from ATCC #10231-D)
- Strands of DNA template hybridized to the primer are attached to beads and suspended in wash buffer
  - Streptavidin M280 Dynabeads
- Enzyme mix:
  - Polymerase + ATP sulfurylase + luciferase + wash buffer
- Reagent mix:
  - dNTP + APS + luciferin + wash buffer + beads
- Combine enzyme mix and reagent mix
  - If correct base, luminescence proportional to number of sequential common bases
- Wash beads and repeat

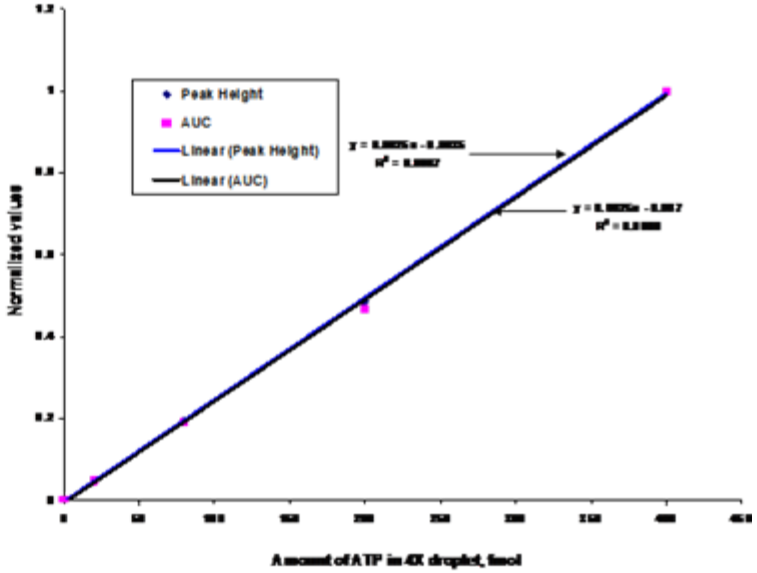
# Pyrosequencing Protocol



# Calibration and Sensitivity

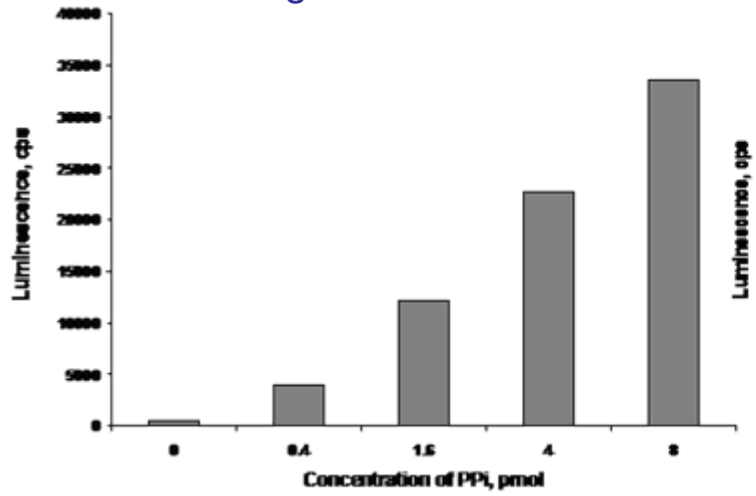
- Possible to detect 100 amol of ATP
- Could sequence DNA attached to single bead in 1pl droplet

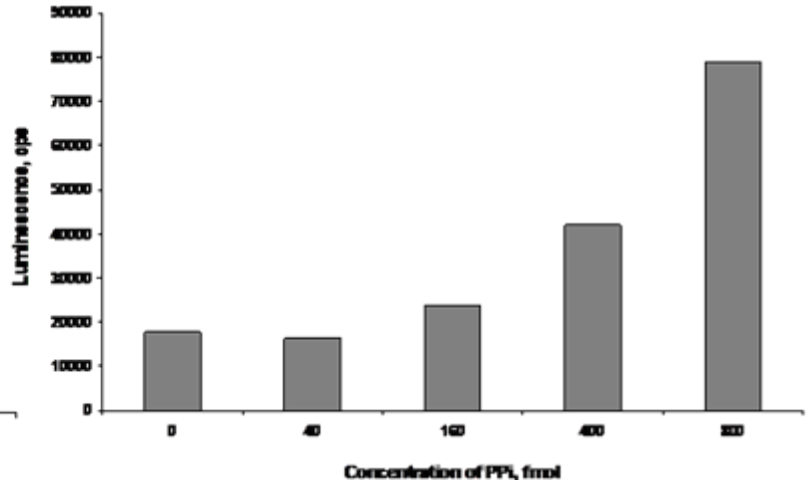


# Issues

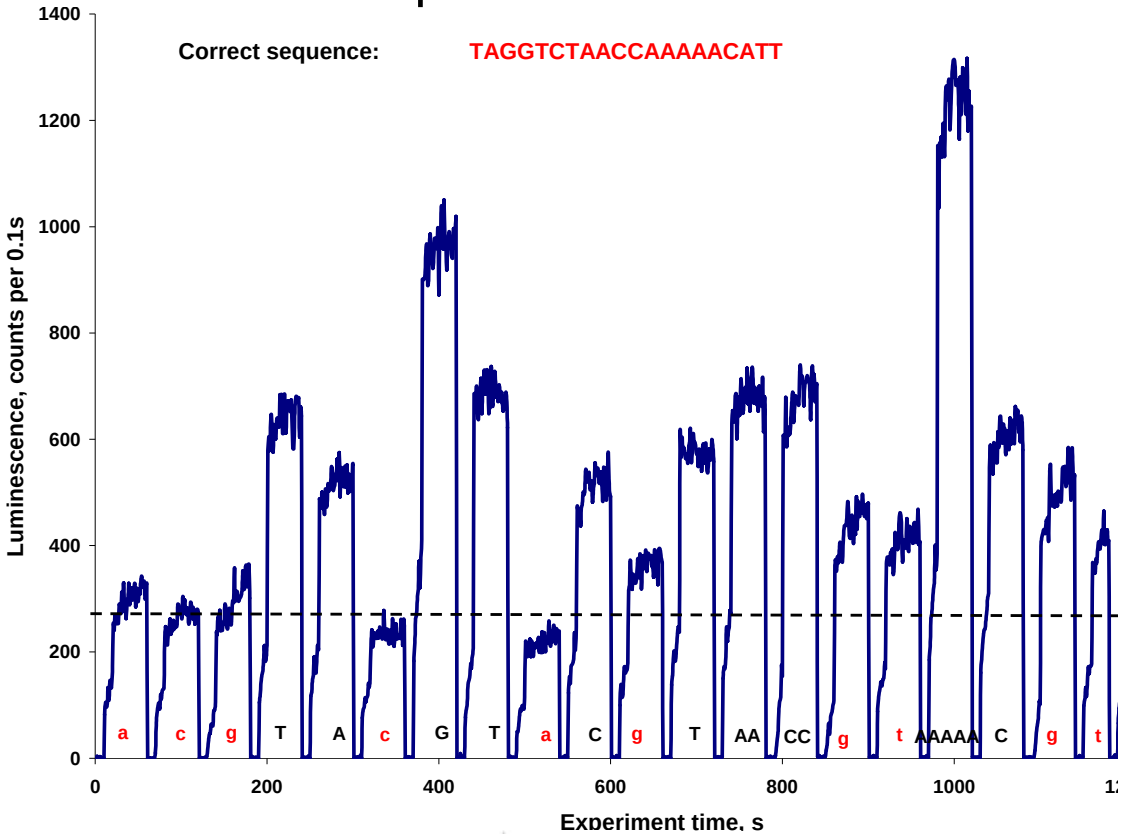
- PPi contamination sets high background
  - chip, reagents, oil
  - Potentially every material used in chips
- Reduced to acceptable levels:
  - Improved chip fab processes
  - Chip cleaning
  - Coatings



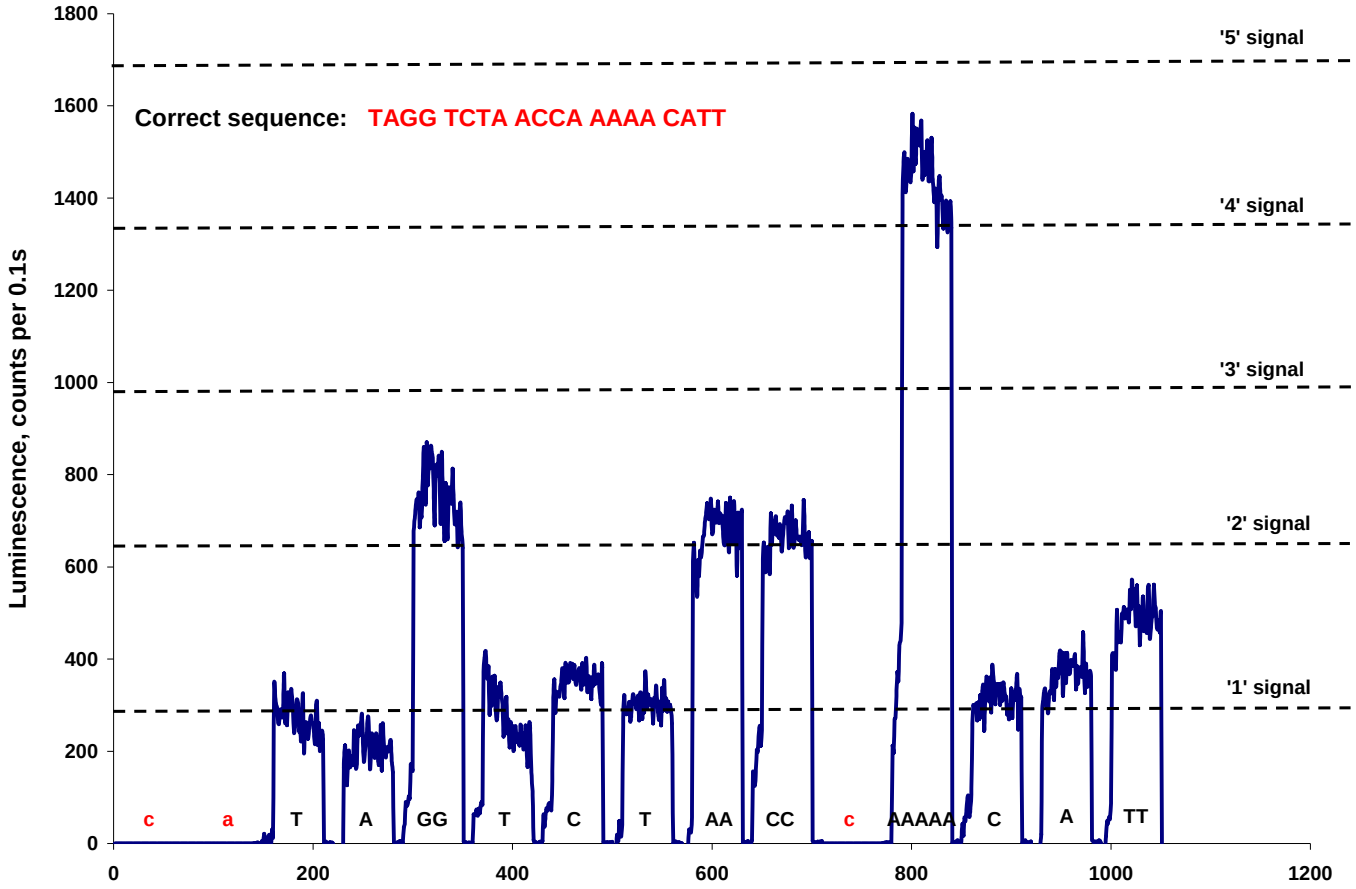



# Results

- 17-bp sequencing of 211-base long C.albicans DNA template with 20-base primer attached



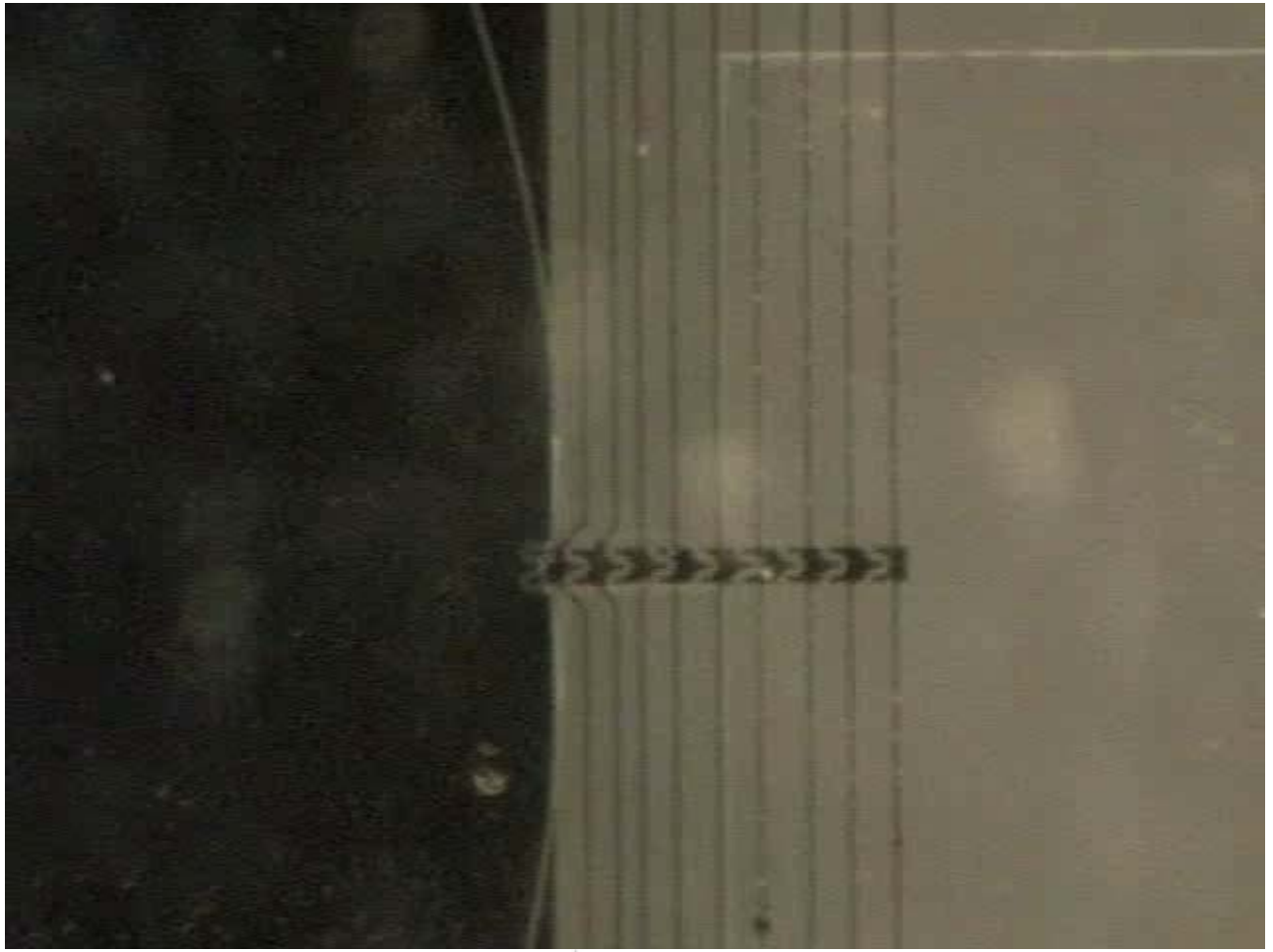
# Homopolymer Fidelity



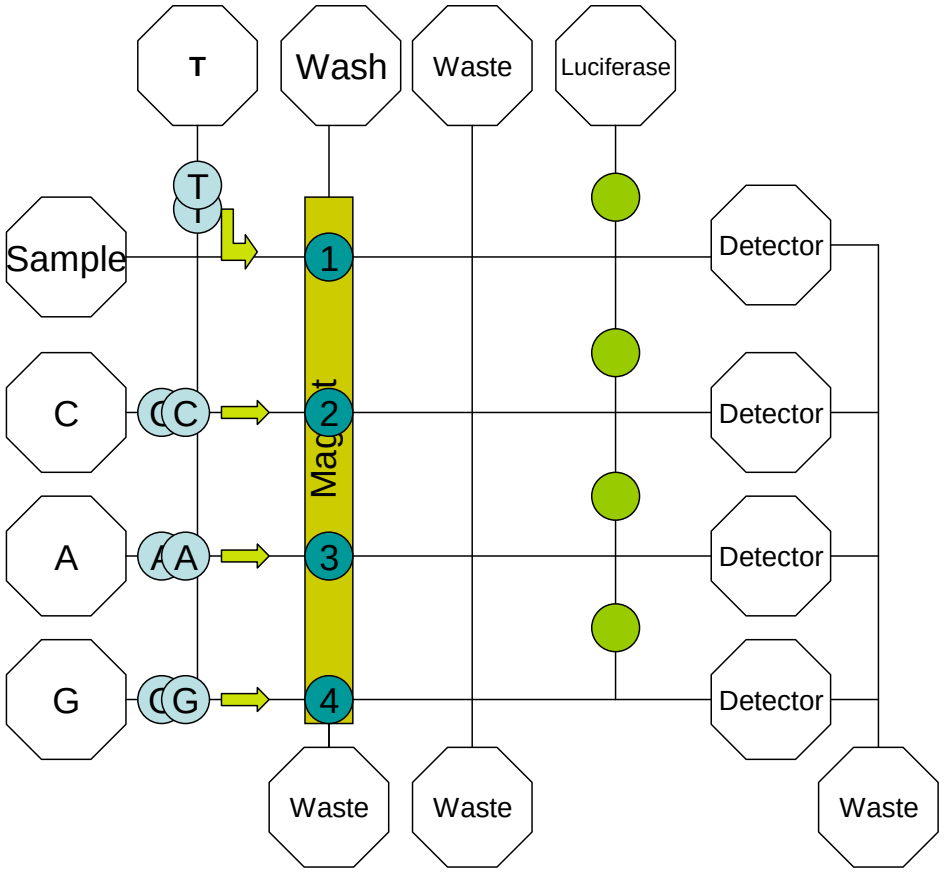
# What's Next?

- Demonstrate continuous synthesis reaction chemistry on immobilized DNA
  - Improve throughput
  - Transport of by-products to a remote array for detection
  - 350 base pair read
  - Scale chemistry to picoliter range
- Determine read length and throughput limits
  - Adaptive reagent delivery with feedback control
  - Simulation
  - Parallel reactions

# 35 Picoliter Droplet Dispensing



# Look ahead, Voting, Chain Washing



# Summary and Conclusions

- Pyrosequencing assay successfully performed on ALL's electrowetting chip platform
- Expected sensitivity is DNA on single-bead in pl droplet
- Demonstrated that the electrowetting platform can be scaled from droplets of hundreds of nanoliters to tens of picoliters
- Demonstrates that complex chemistry can be performed on microfluidic chips
  - Requires stable, reproducible platform
  - No cross-contamination
  - Programmable operation to manage complexity

# Acknowledgement


- NHGRI grant 1R01-HG004354-01: “Continuous Sequencing-By-Synthesis Based on a Digital Microfluidic Platform”