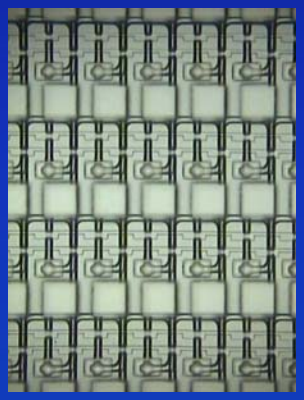
The Promise of Complex Microfluidic Circuitry

APS March Meeting
Opportunities in Biological Physics Workshop
March 4th, 2007



Robert C Jones
Executive Vice President
Research and Development
Fluidigm Corporation

Fluidigm

Abstract from Program

- Just as electronic integrated circuits dramatically reduced the size of electronic equipment, integrated fluidics circuits (IFCs) are uniquely suited to do the same for life science. In these devices, liquid and control channels are molded into polydimethylsiloxane (PDMS) layers using patterns of photoresist on silicon wafers. Pressure is used to control valves and drive liquids to perform desired reactions. A very wide variety of devices, including valves, pumps, mixers, reaction chambers, separation columns and detection cells, can be fabricated at very high density to work together in a single chip. These products reliably perform thousands of biological reactions in a 3cm x 3cm device. To make these devices, research and development teams have addressed challenges across the fields of molecular biology, chemistry, optics, fluidics, materials science, engineering, and software. Due to the highly interdisciplinary nature of this work, scientists with physics training are needed to drive the technology forward. As devices for single cell and single molecule analysis are developed, challenges at the interface between biology and physics offer unique opportunities for physicists to make quantitative contributions to biology.

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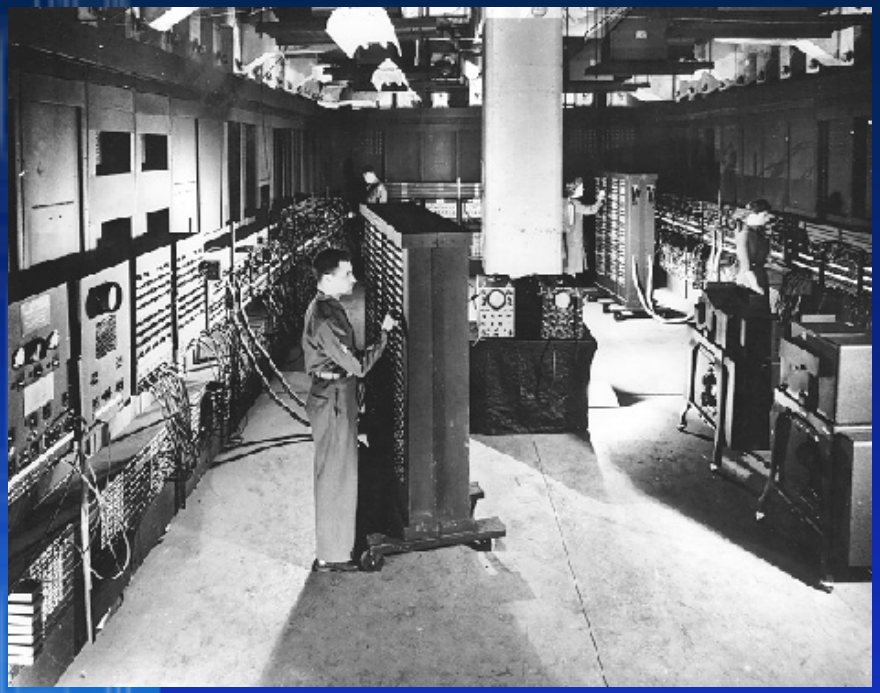
The IC Revolutionized Electronics



1952: The ENIAC
The "Tyranny of Numbers"

2005: The Itanium 3
>150,000 ENIACs

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Miniaturization and Integration Fueled the Electronics Revolution

Vacuum tube

Transistor

Integrated Circuit

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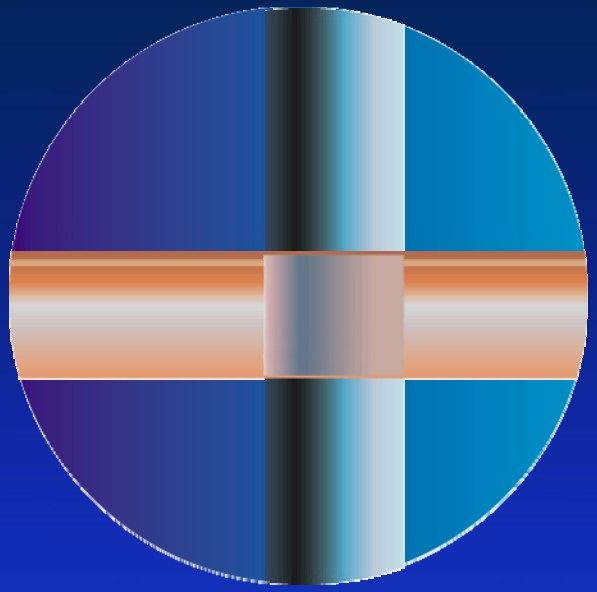
Miniaturization for Fluidics

Transistor

Enabled integrated *electronic* circuit

Fluidigm's nano-flex™ valve:



Enables integrated *fluidic* circuit

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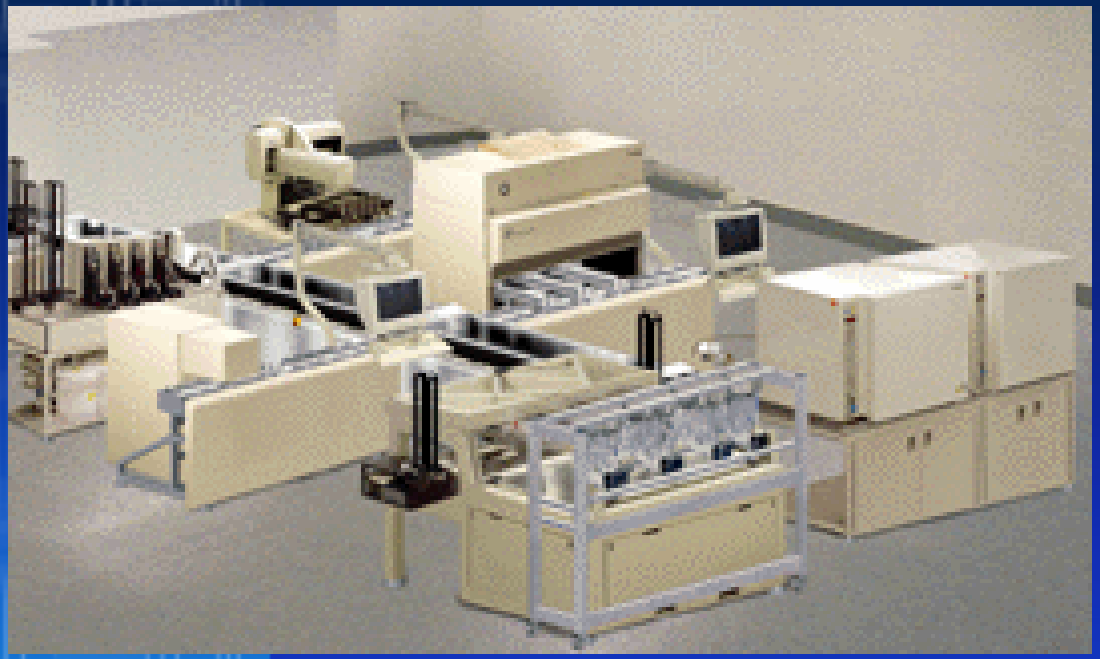
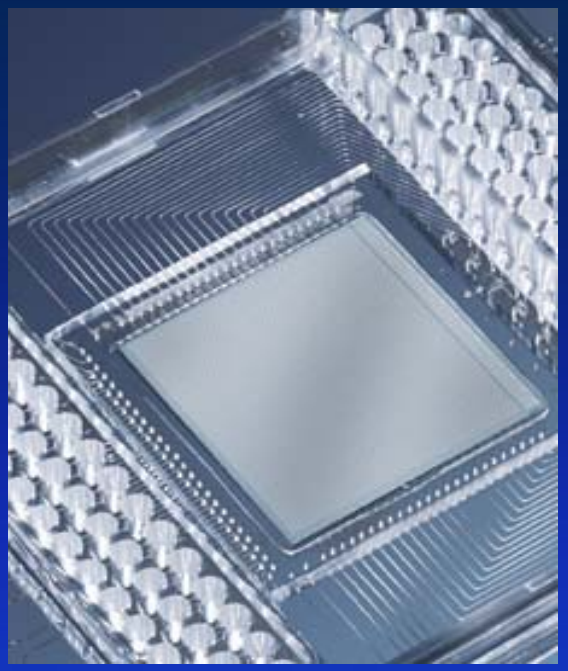
Fluidigm's Premise: Integrated Fluidic Circuits (IFCs) Could Revolutionize Biology





2005: The "Tyranny of Pipetting:" A $6B Industry

IfCs bring non-linear economic benefits and unleash new physics

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Nano-Flex™ Valve Open

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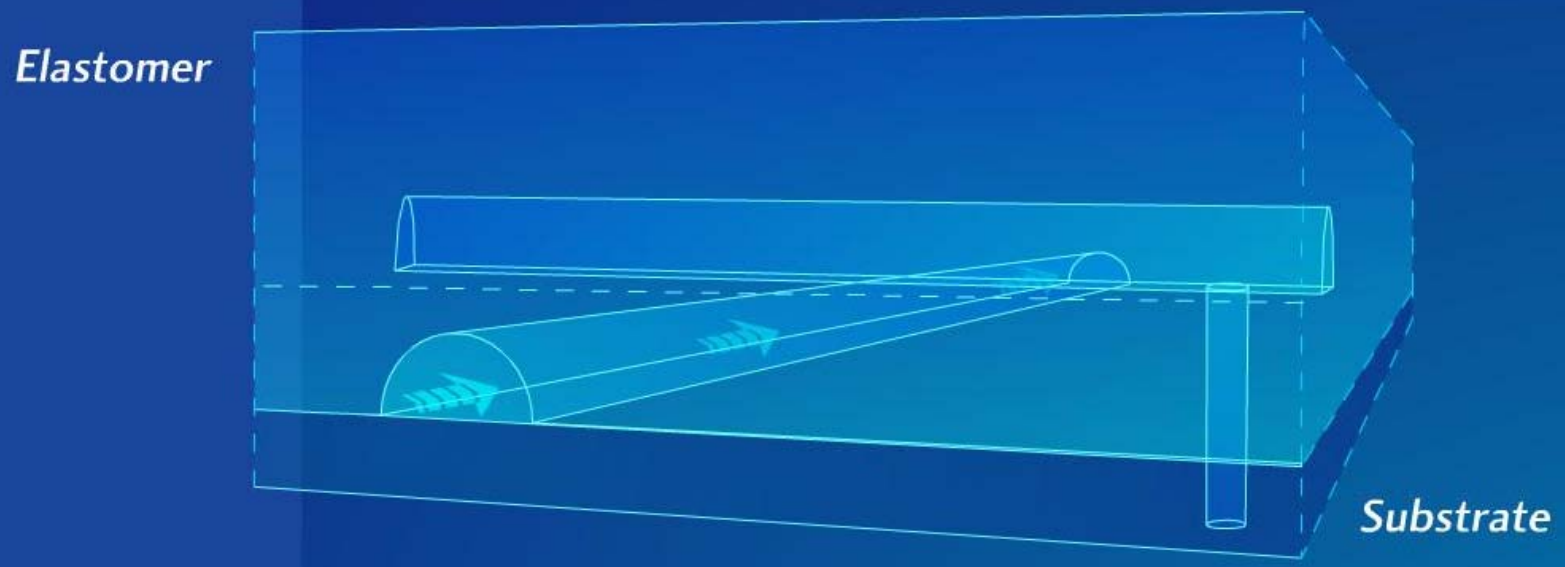
The NanoFlex™ Valve

A NanoFlex valve is formed where a channel in the upper (control) layer overlaps a channel in the bottom (fluid) layer.



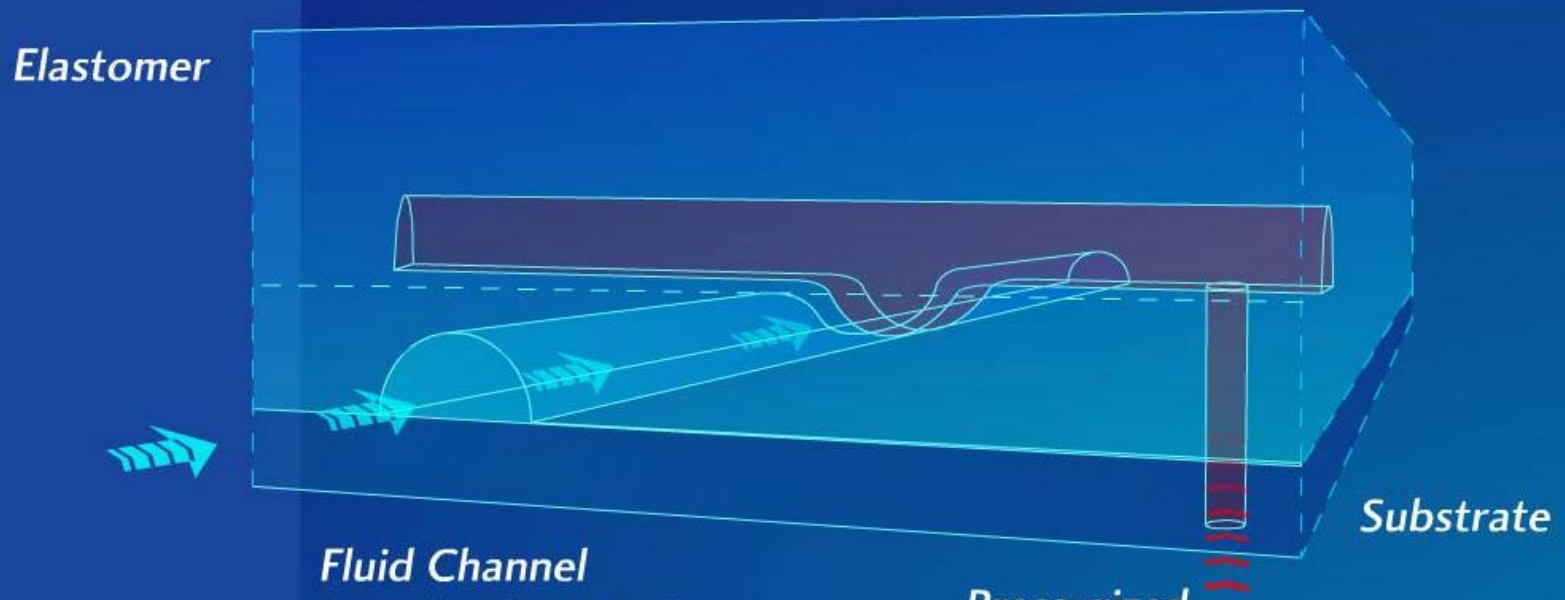
VALVE – OPEN

Nano-Flex™ Valve Closed

F L U I D I G M

The NanoFlex™ Valve

Air pressure is applied to the channel in the control (top) layer from an external source. This pressure causes the thin membrane between the layers to deflect into the fluid (bottom) channel, pinching off the flow of fluids.



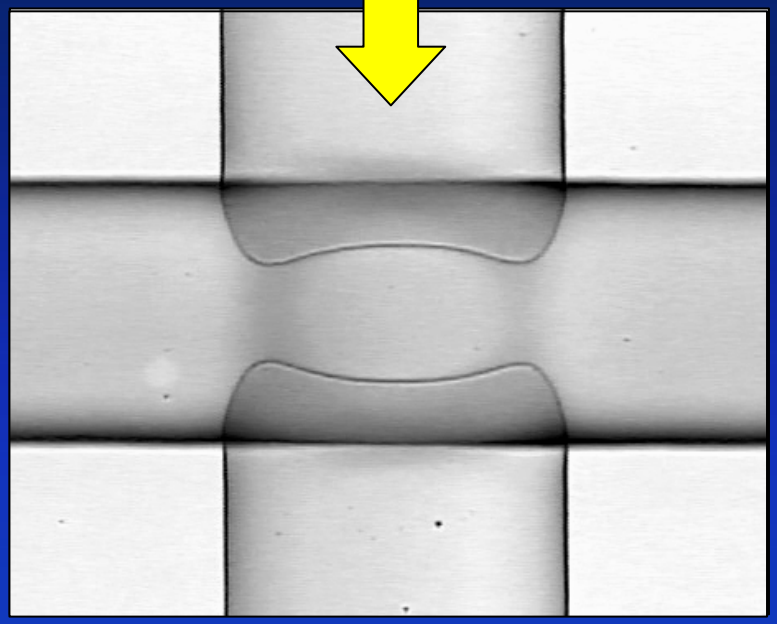
VALVE – CLOSED

Miniaturization for Fluidics

Fluid Channel

Closed

Control Channel



~50 microns

~100 microns

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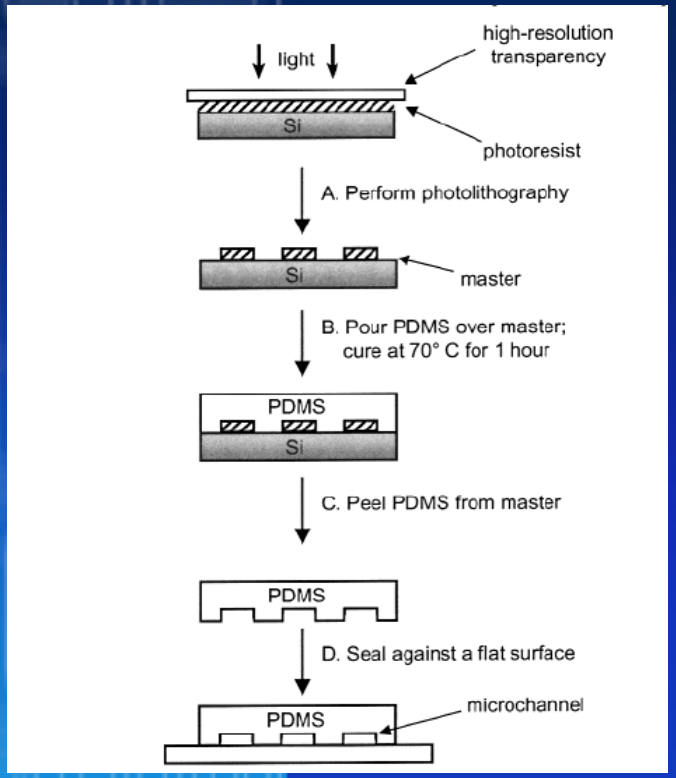
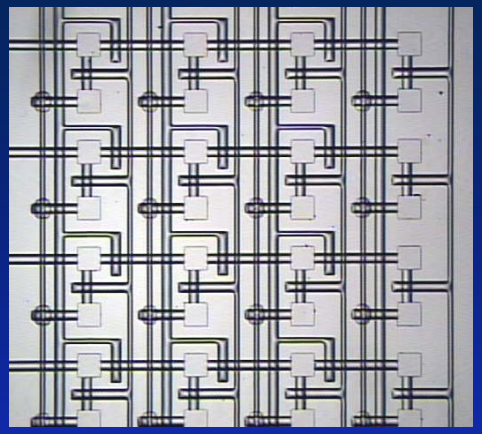
Multi-Layer Soft Lithography

Soft Lithography

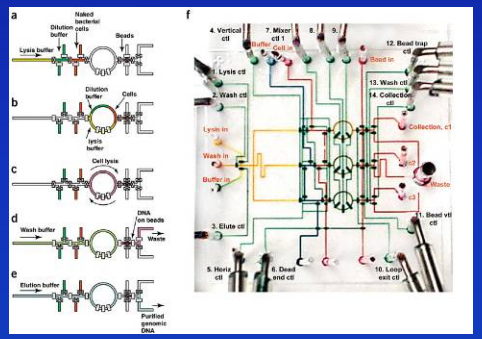


Advantages of *Multi-Layer* Soft Lithography

- Rapid Prototyping
- Blind filling
- Accurate metering
- Bio-compatible



- Many kinds of devices
  - Valves
  - Pumps
  - Mixers
  - Purification Columns
  - Reaction chambers



Hong, J., Studer, V., Hang, G, Anderson, W.F., Quake, S., A nanoliter-scale nucleic acid processor with parallel architecture. Nature Biotechnology 22, 435 - 439 (2004)

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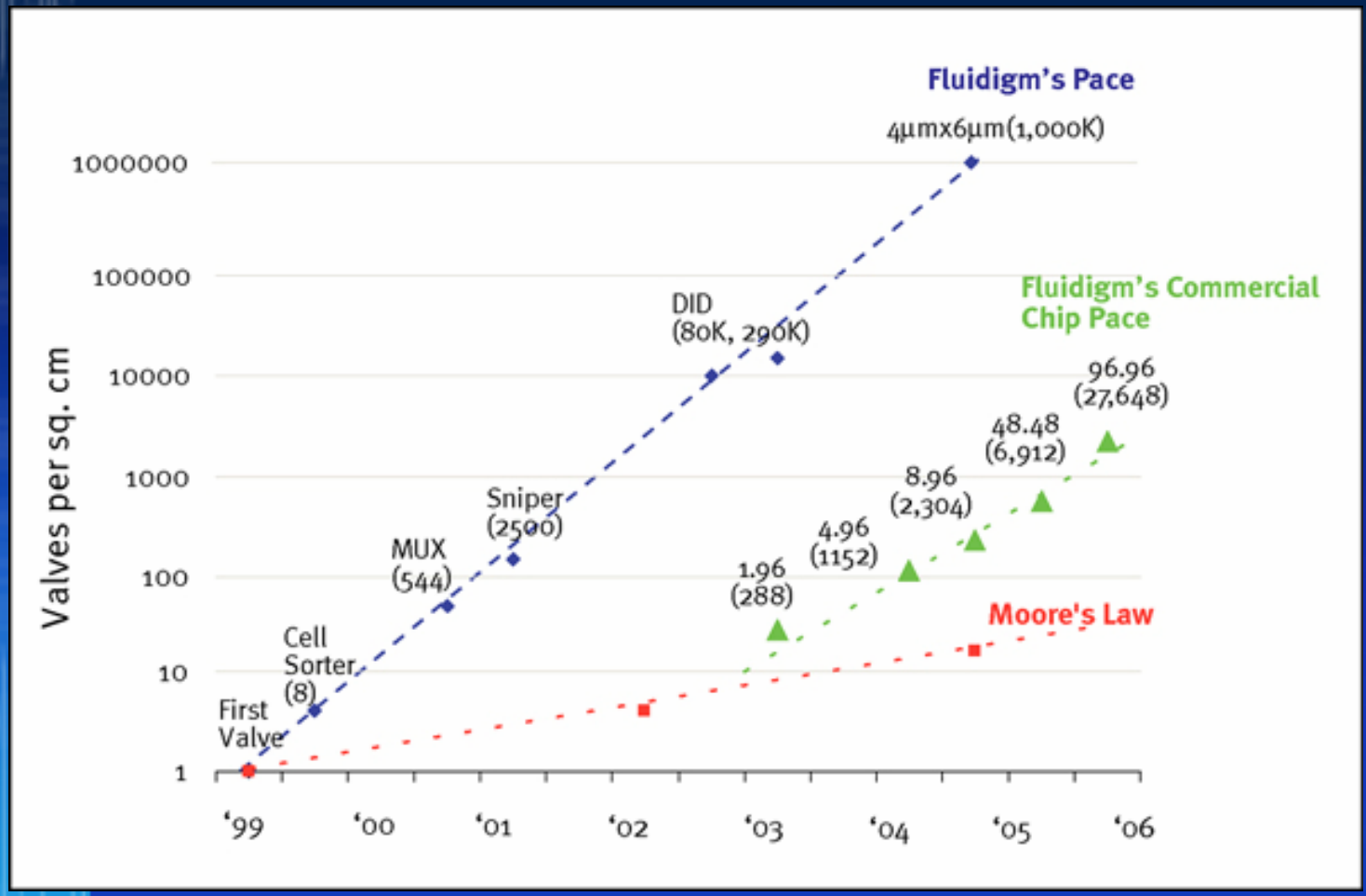
Moores law



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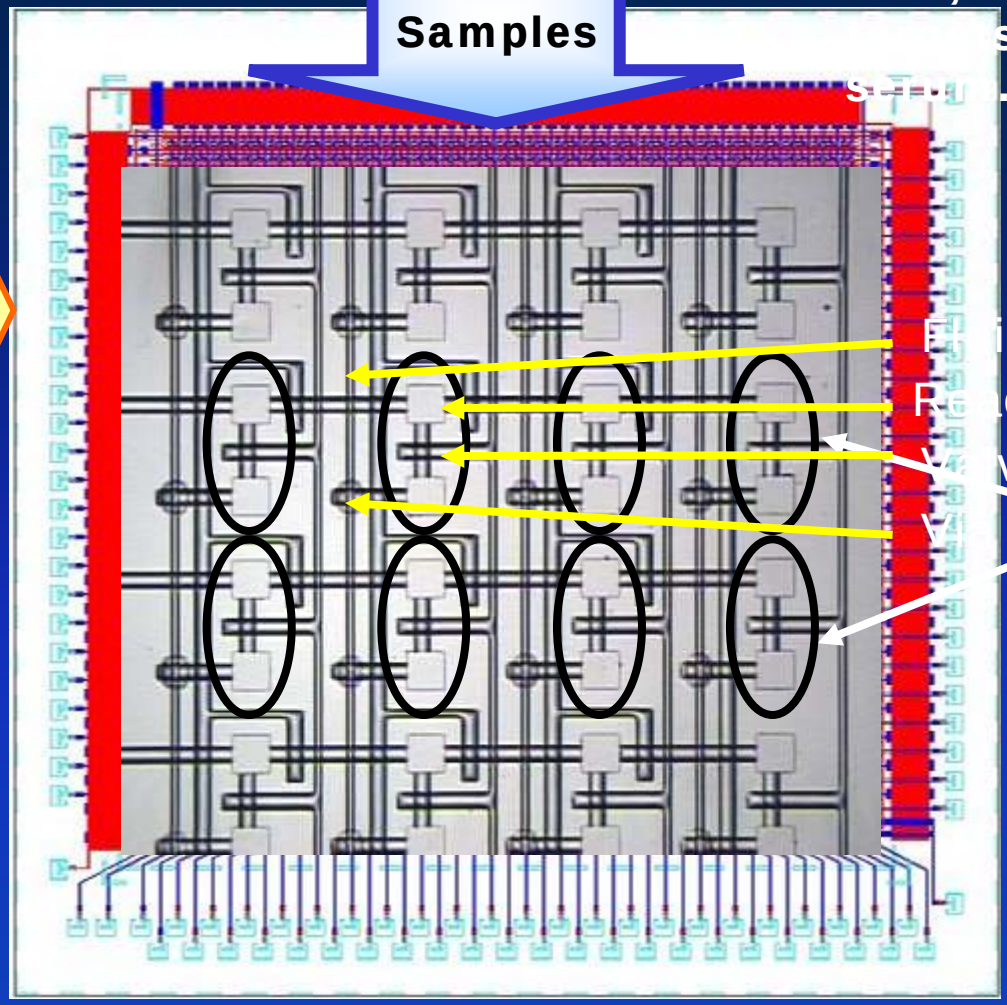
The Dynamic Array: *Any*

M
Samples

Proteins, DNA, CDNA, RNA, complexes, drug s, viruses, ...

N
Assays



id Channel
ction Chamber
ve

NxM discrete nano-liter reactions

Crystallization reagents, PCR, Taqman, Invader, Eclipse, drugs, antibodies, probes ...

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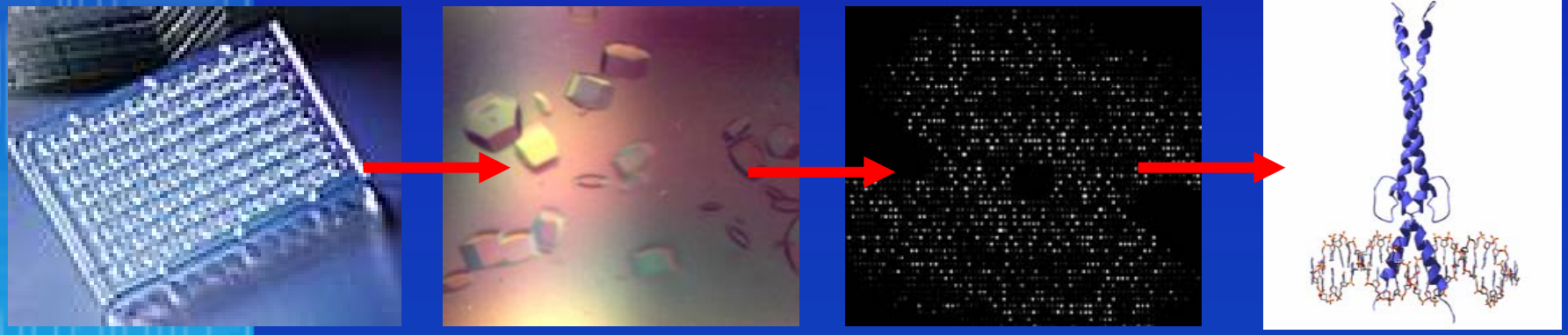
Protein Crystallography: A Technique to Discover Protein Structure

- Atomic Resolution from X-ray Diffraction
- Protein Structure/Function
- Rational Drug Design
- Structural Genomics



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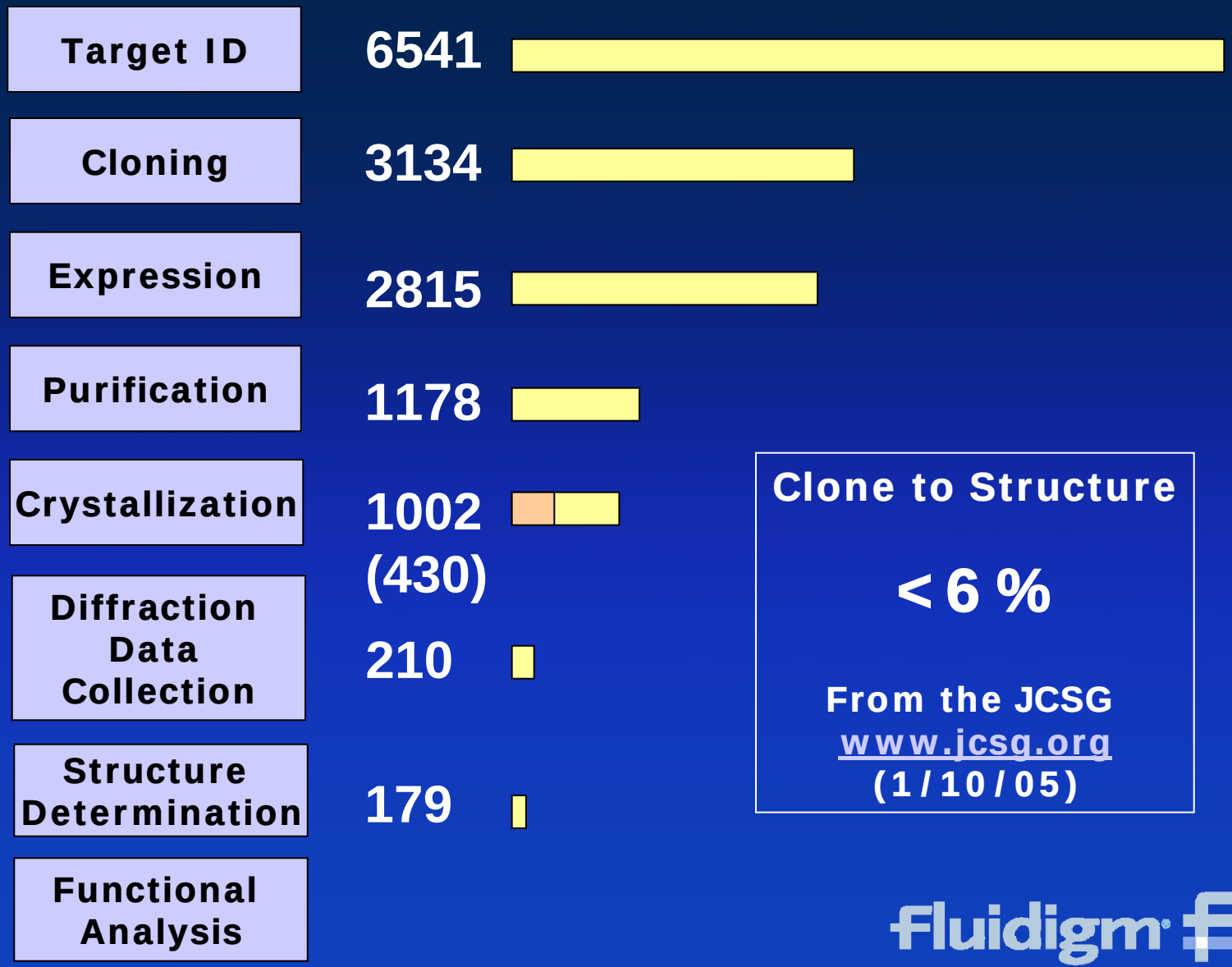
Structure Discovery



Clone to Structure

< 6 %

From the JCSG
www.jcsg.org
(1/10/05)

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MicroFluidics Automates and Enables Structure Discovery with Novel Fluid Physics

- Standard methods utilize robots of ever-increasing complexity to replace pipetting; format of experiment has remained essentially unchanged.
- Microfluidics allows efficient and scalable automation at the nanoliter level which conserves expensive and difficult to produce proteins
- Novel fluid physics at this scale allow new and more efficient nucleation and growth kinetics for crystals.

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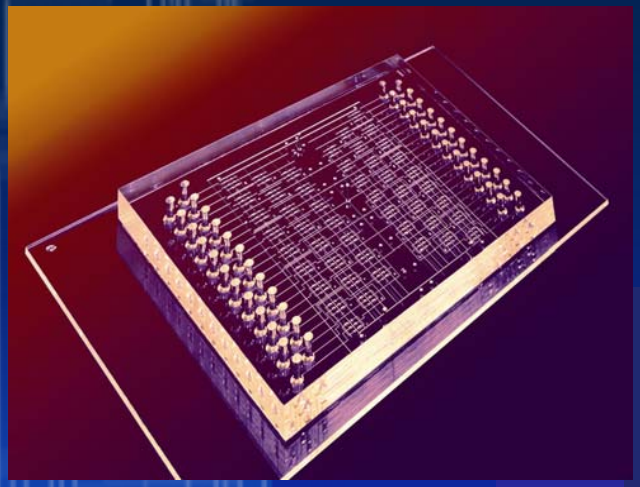
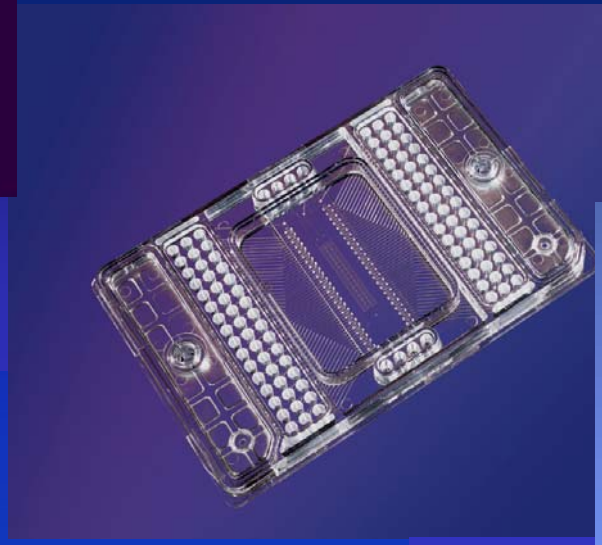
Increase in Throughput

1.48 Chip - 2001



1.96 Chip - 2003



4.96 Chip - 2004

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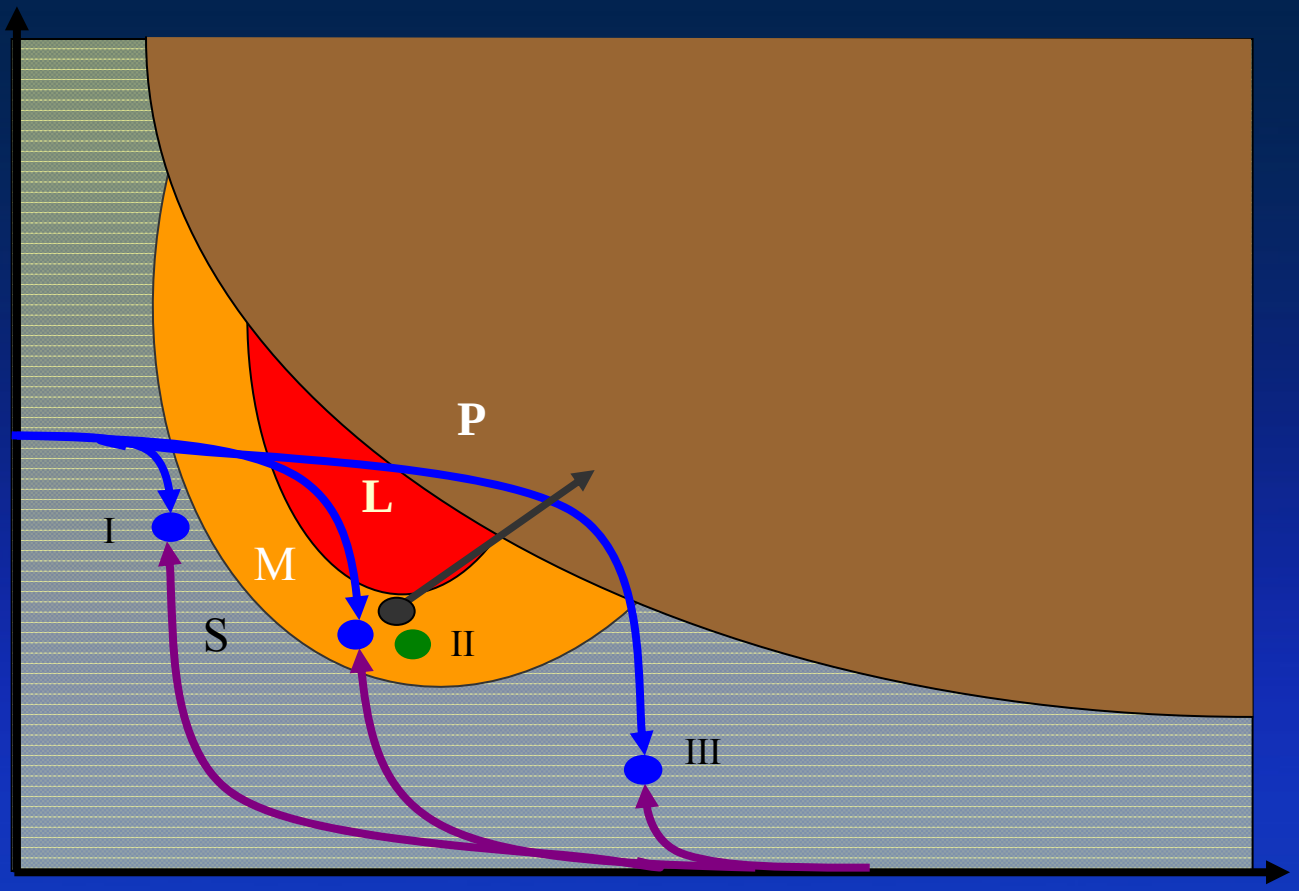
Efficient Exploration of Phase Space by Free Interface Diffusion



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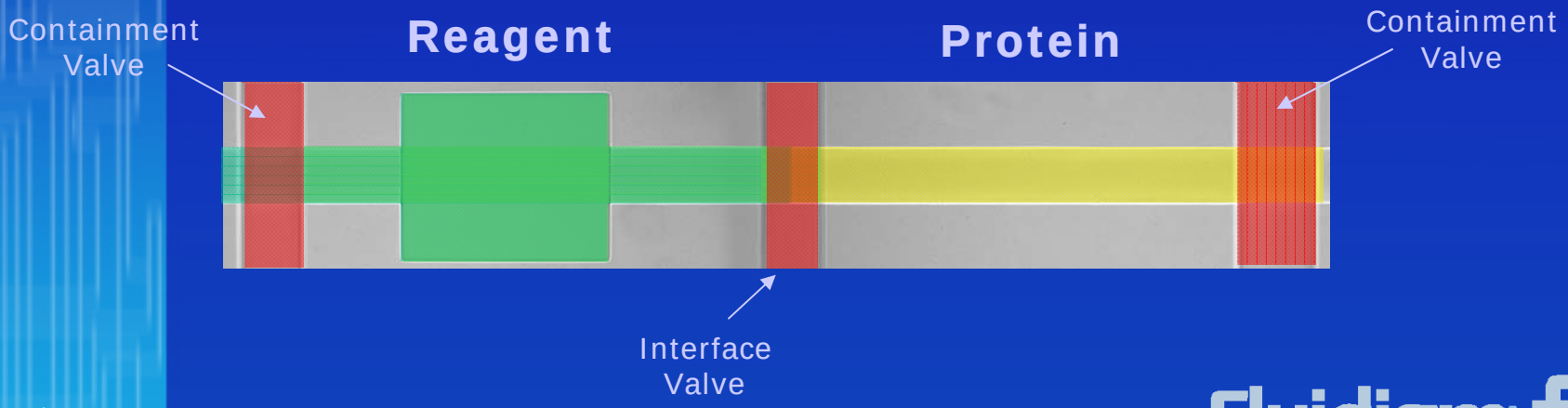
Microfluidic Free-Interface Diffusion

- Protein and reagent are loaded into separate wells separated by an interface valve
- Opening of the valve permits diffusion of protein and reagent
- After diffusion, permeable PDMS allows gradual dehydration



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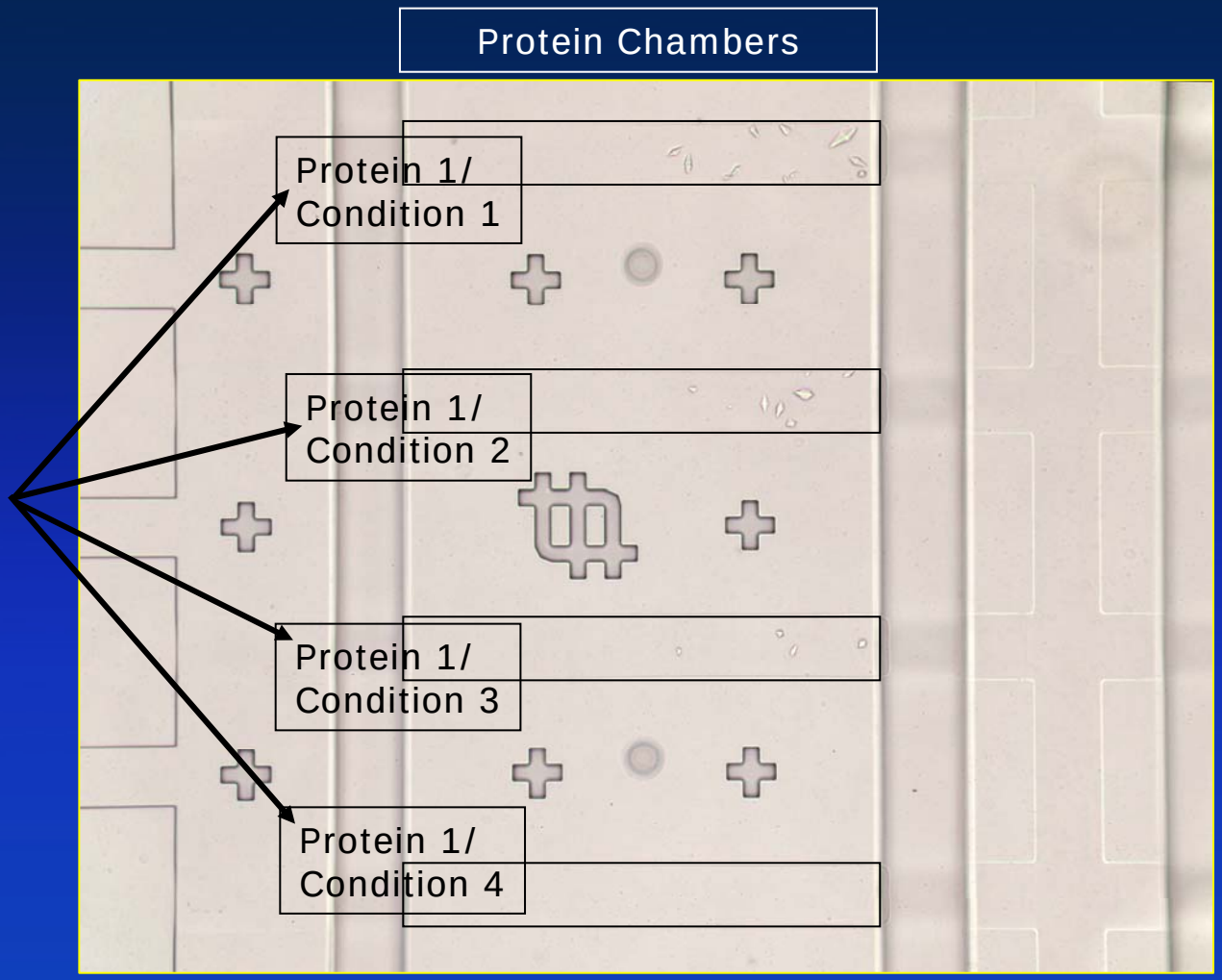
Chip layout



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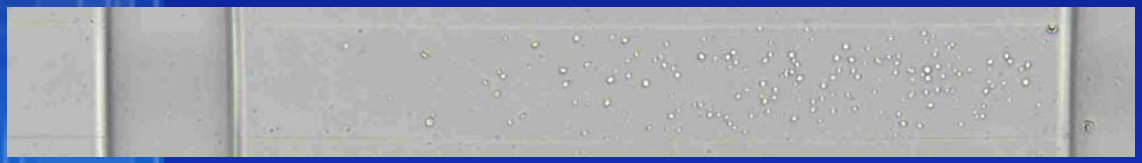
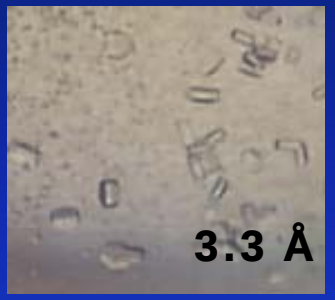
Crystal quality vs resolution

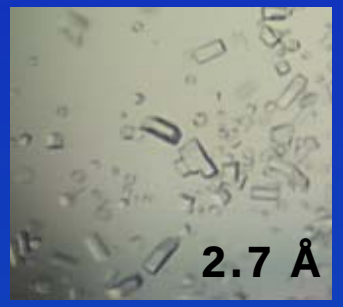
- MS001: 22kDa monomeric calcium binding protein (chicken) @ 20mg/ml

- MS132: 67kDa hexameric LipidA modifying enzyme (S. typhimurum)@ 8mg/ml





- WW002: 30kDa AAA Atpase domain (mouse) @ 6 mg/ml



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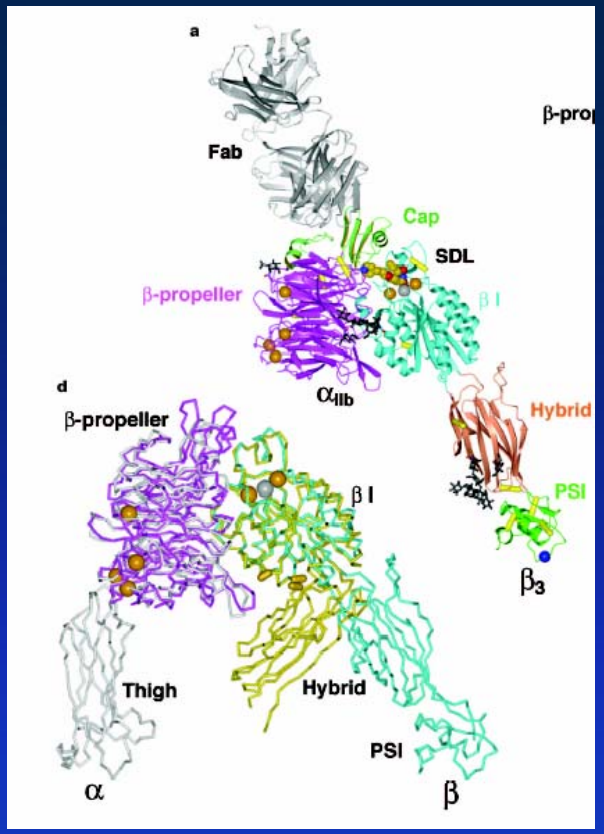
Membrane protein: Alpha Integrin



Xiao, Tagaki, Coller, Wang, and Springer, Nature, **432**, 59 (2004).

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External Validation (SPinE)

- **The TOPAZ system was successful in:**
  - **Crystallizing all 5 proteins that crystallized using vapor diffusion**
  - **Crystallizing 3 of 9 proteins that did not crystallize in vapor diffusion**
  - **Crystallizing 6 of 13 proteins that had not crystallized previously**

Sample	Previously Crystallized ?	Fluidigm Success (96 Screens)	Vapor Diffusion Success (384 Screens)
1	No	0	0
2	Yes	12	
3	Yes	9	
4	Yes	6	
5	No	0	0**
6	No	1	0**
7	No	0	0
8	Yes	4	1
9	Yes	2	4
10	Yes	9	47
11	No	0	
12	No	0	
13	Yes	2	3**
14	Yes	24	
15	No	2	
16	No	1	
17	Yes	3	0***
18	Yes	0	0**
19	No	1*	
20	No	0*	
21	No	2*	
22	No	2*	
23	Yes	7	0
24	Yes	5	9
25	No	0	0
26	Yes	0	0
27	Yes	4	0

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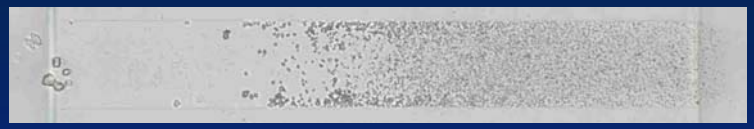
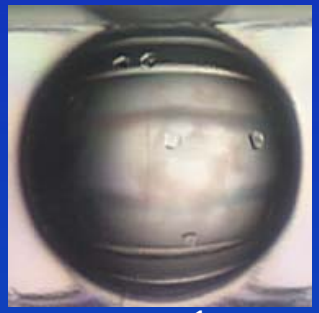
Translation screening hits



Optimization

Small number of
high quality crystals

Translation



1mm

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Translation Validation by 2 Pharmaceutical Customers

Total Number of Protein Samples tested with TOPAZ		
Customer Number	**1**	**2**
Number of Proteins tested	7	8
Number of Co-Crystallization Experiments	5	7
Previous Success in Vapor Diffusion		
Number Tried Previously	4	6
Number Crystallized Previously	3	6
Number of Trials attempted previously		1000+
TOPAZ Success		
Number Crystallized in Screening Chip	5	7
Attempted Translation via Vapor Diffusion	3	4
Translated via Vapor Diffusion	3	4
Translation Success	100%	100%
Attempted Translation via X-Ray Chip	0	7
Translated in X-Ray Chip	0	7
Translation Success	n/a	100%
Number Diffracted	3	6
Number of Structures from TOPAZ	3	2

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Digital Array

- Principle
  - Partition a sample into many parts and treat as independent reactions
  - Detect the difference between 1 and 0 and then count
- Advantages
  - Easier to detect binary differences and then count
  - Reduces competing side reactions
  - Enables absolute quantification in biological systems
  - Isolate and measure a rare portion of a sample that would otherwise be undetectable due to background noise
    - Partition a limited sample into 1000 partitions and 1:100,000 becomes 1:100 for those chambers containing a target molecule

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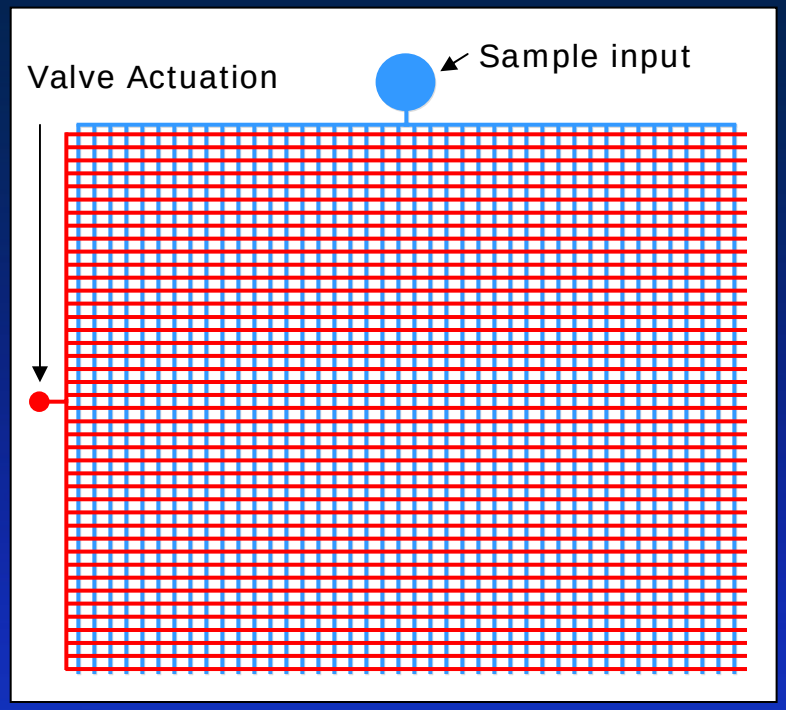
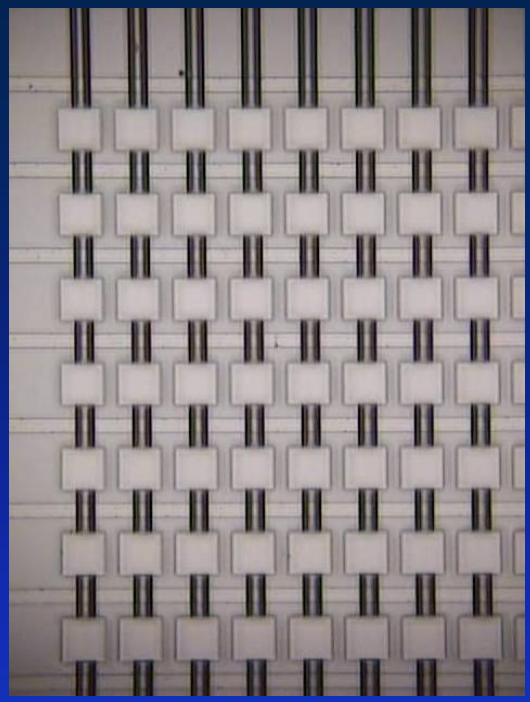
Digital Array Chip Partitioning





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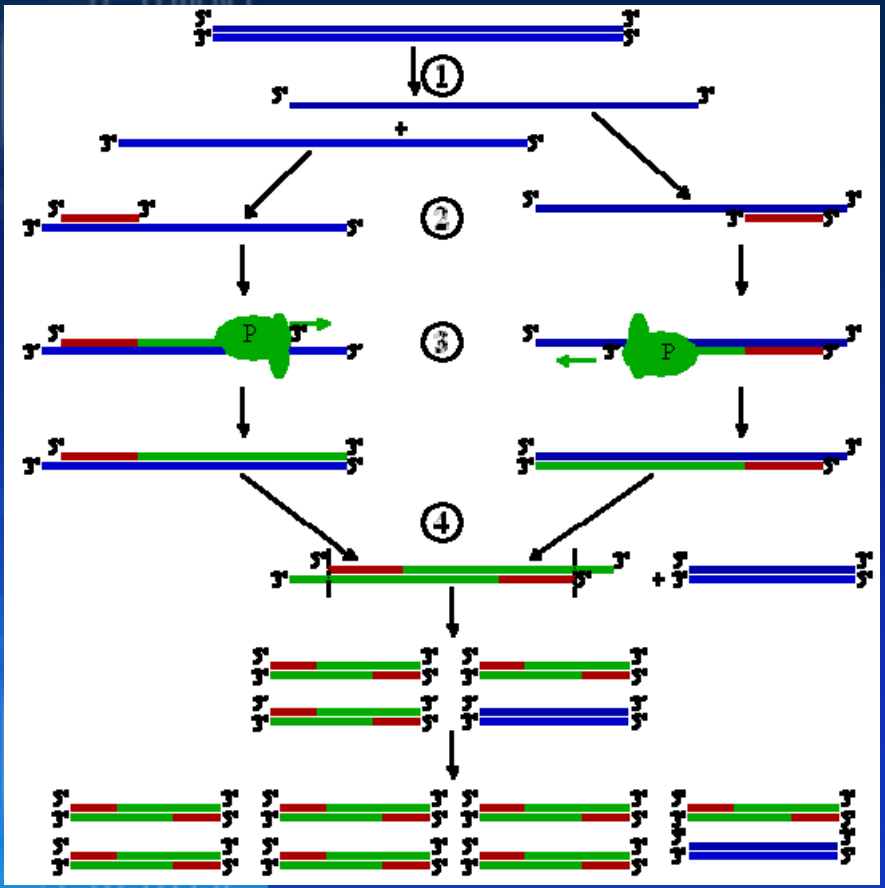
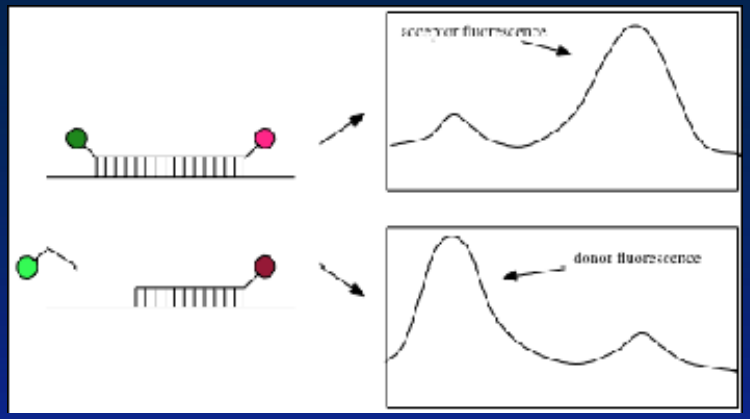
Detection by Real-Time PCR





- Selective target amplification
- Detection with FRET probes

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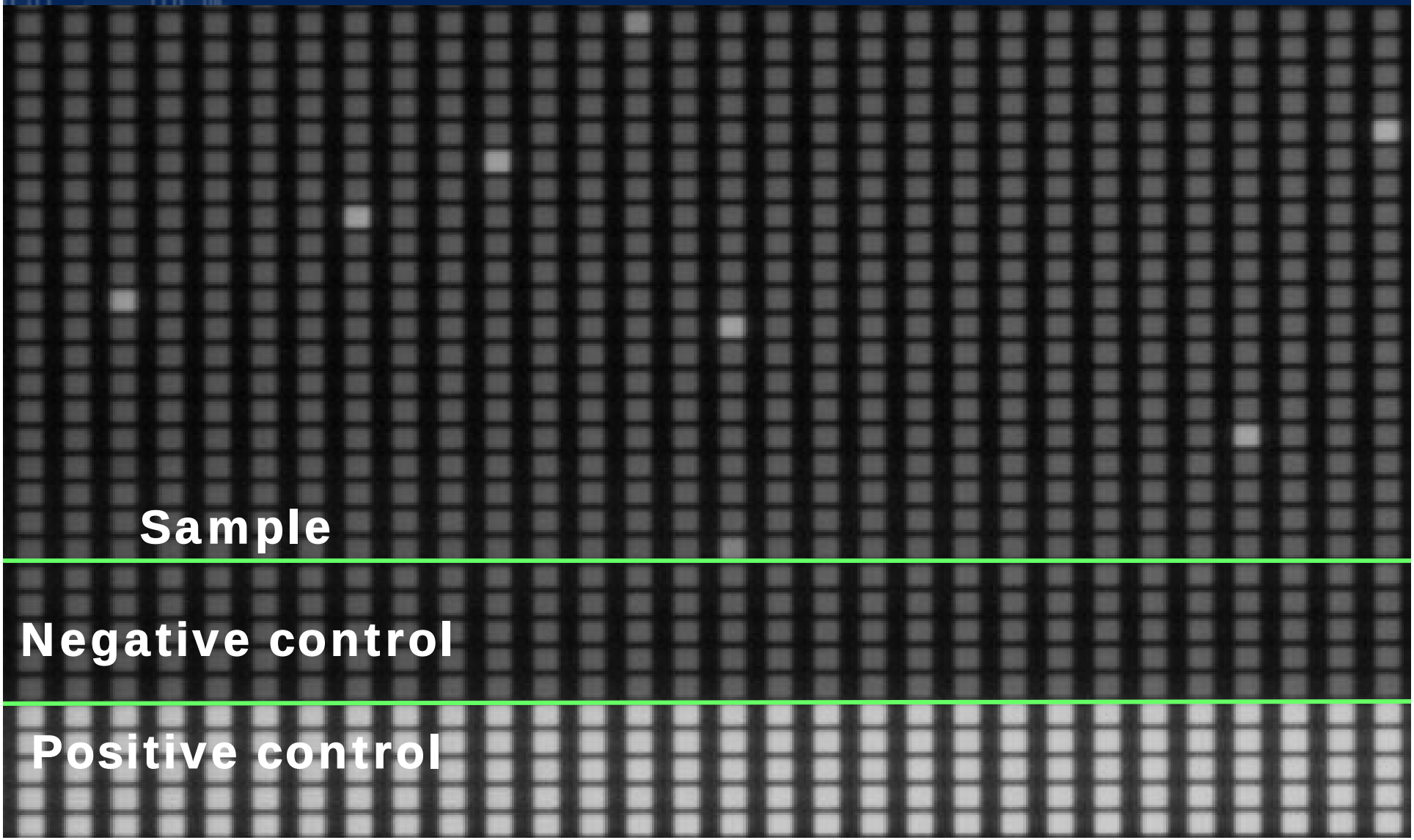
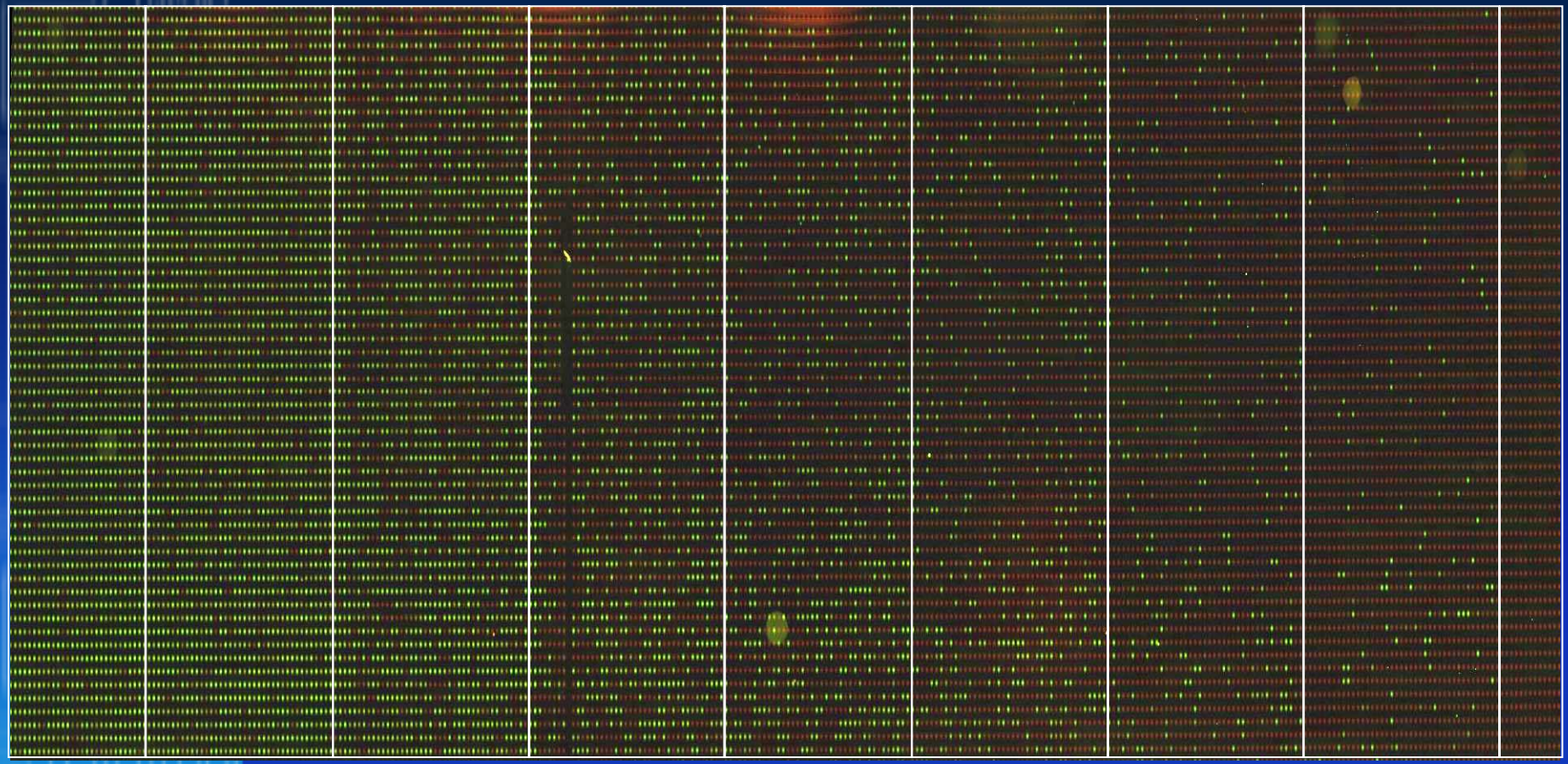
Real-time PCR in Digital Array



2400 x 100pL partitions x 8 samples in 17mm x 55mm



5.2 3.5 1.7 0.9 0.5 0.4 0.2 0.1 0

Number of target molecules per partition

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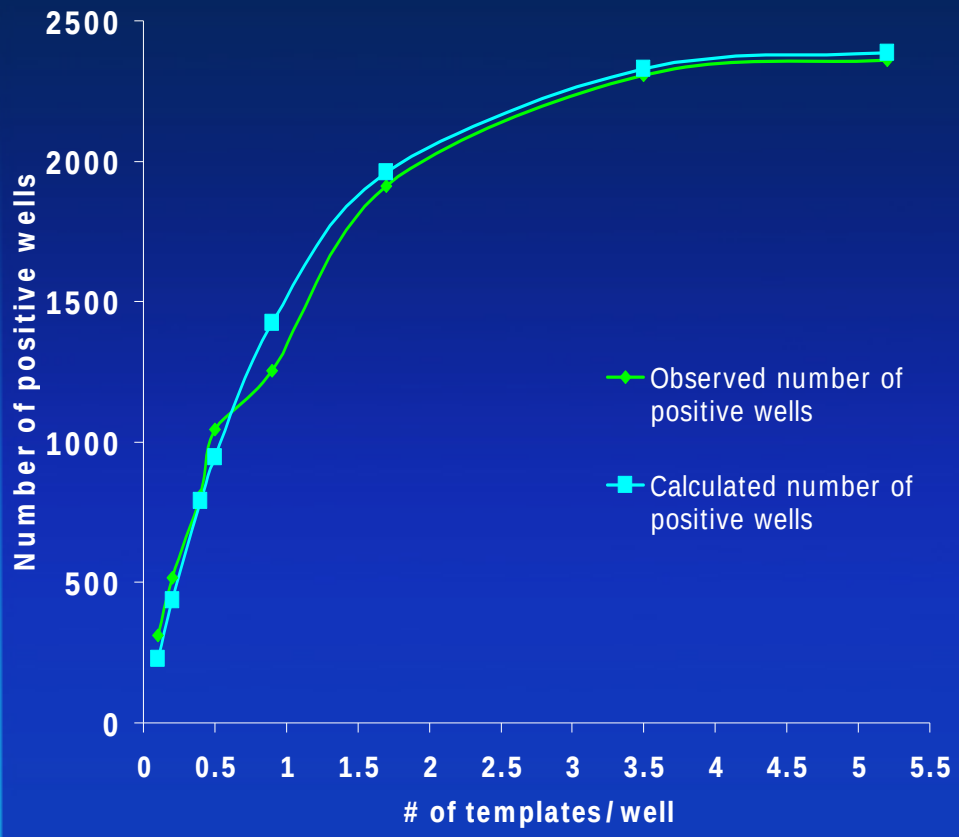
Efficient template amplification in 100pL wells



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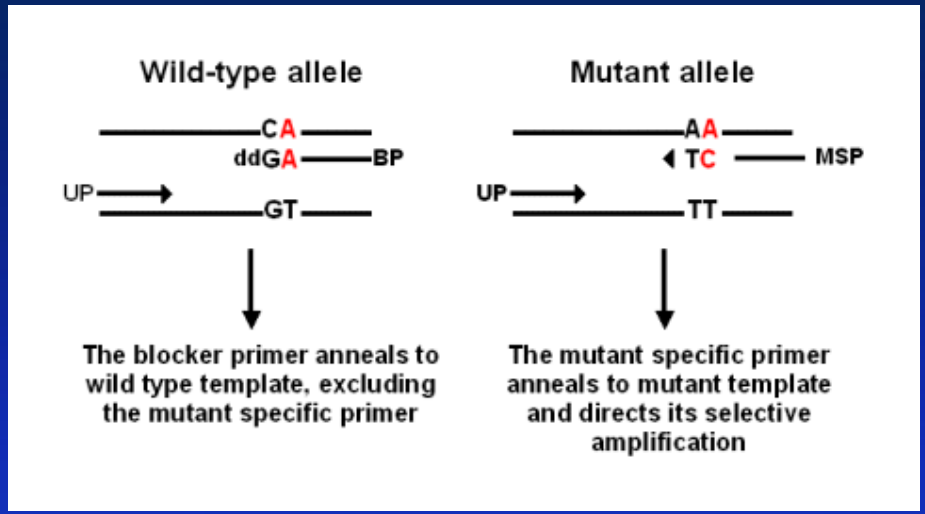
Rare Mutation Detection

- Rare single base mutations challenge PCR's discrimination
- Discrimination by enhanced by allele specific PCR



- Digital Isolation in effect reduces concentration of wildtype by a factor equal to the number of partitions
- *1 mutant per 100,000 wild time becomes 1:100*

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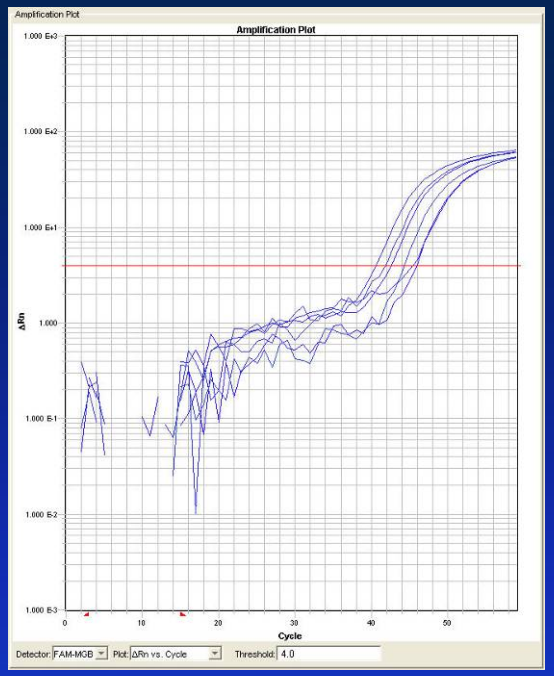
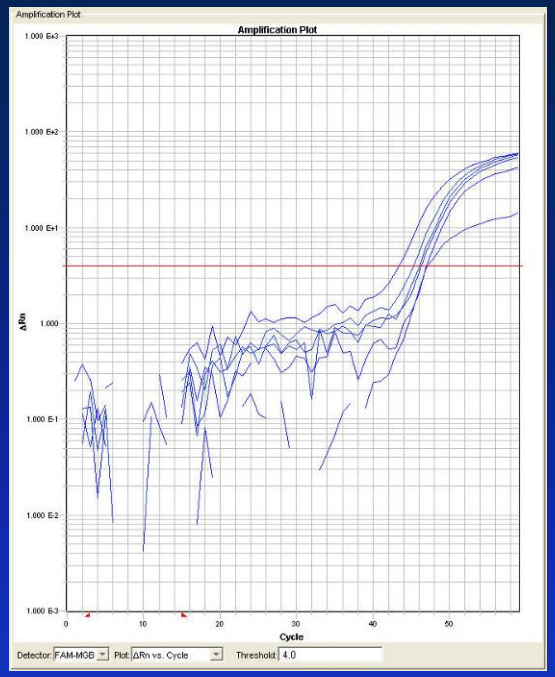
Point Mutation Sensitivity

Mutant Dilution of 1:20,000 in wild type DNA

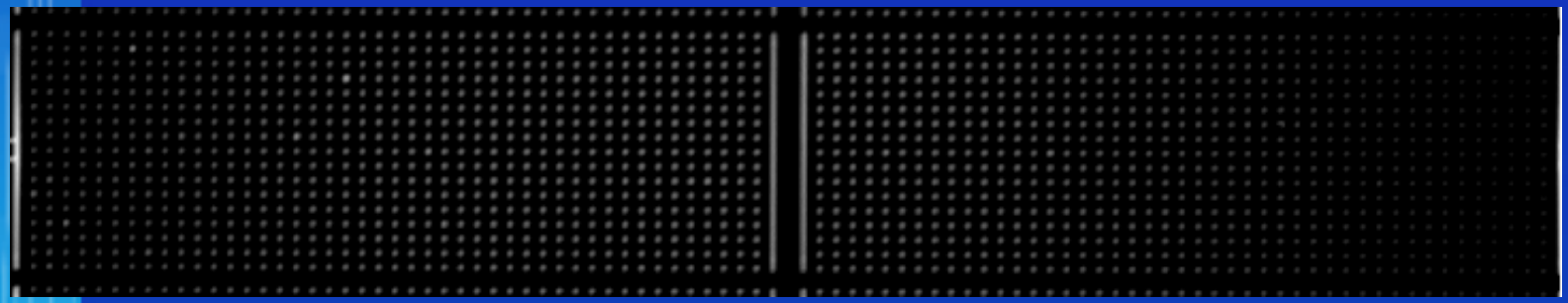
Wild type DNA alone

ABI 7900





Digital Array



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Abl-tyrosine kinase domain mutation detection in chronic myeloid leukemia

- Abl-tyrosine kinase inhibitors have become first line treatment for chronic myeloid leukemia (Imatinib)
- Earlier detection of drug resistance could inform the switch to alternate therapies
- Current sequencing based methods have about 25% sensitivity

- Jerald Radich, M.D. Fred Hutchinson Cancer Research Center
- Vivian Oehler, M.D.
- Dr. Frank McCormick UCSF Medical Center
- Suchitra Ananthnarayan

APS March Meeting
Denver, Colorado
March 5, 2007

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Abl-tyrosine kinase domain mutation detection in chronic myeloid leukemia

- Goal: Detect and quantify a minor population of a point mutation using the Digital Array
- Mutation-specific PCR assays were developed for: G250E, Q252H (2 substitutions), Y253F, E255K, E255V, and T315I
- Tools: Bcr-Abl point mutated plasmids, Bcr-Abl point mutated BaF3 cell lines, and mutation specific primers, and PCR

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Structure and assay placement

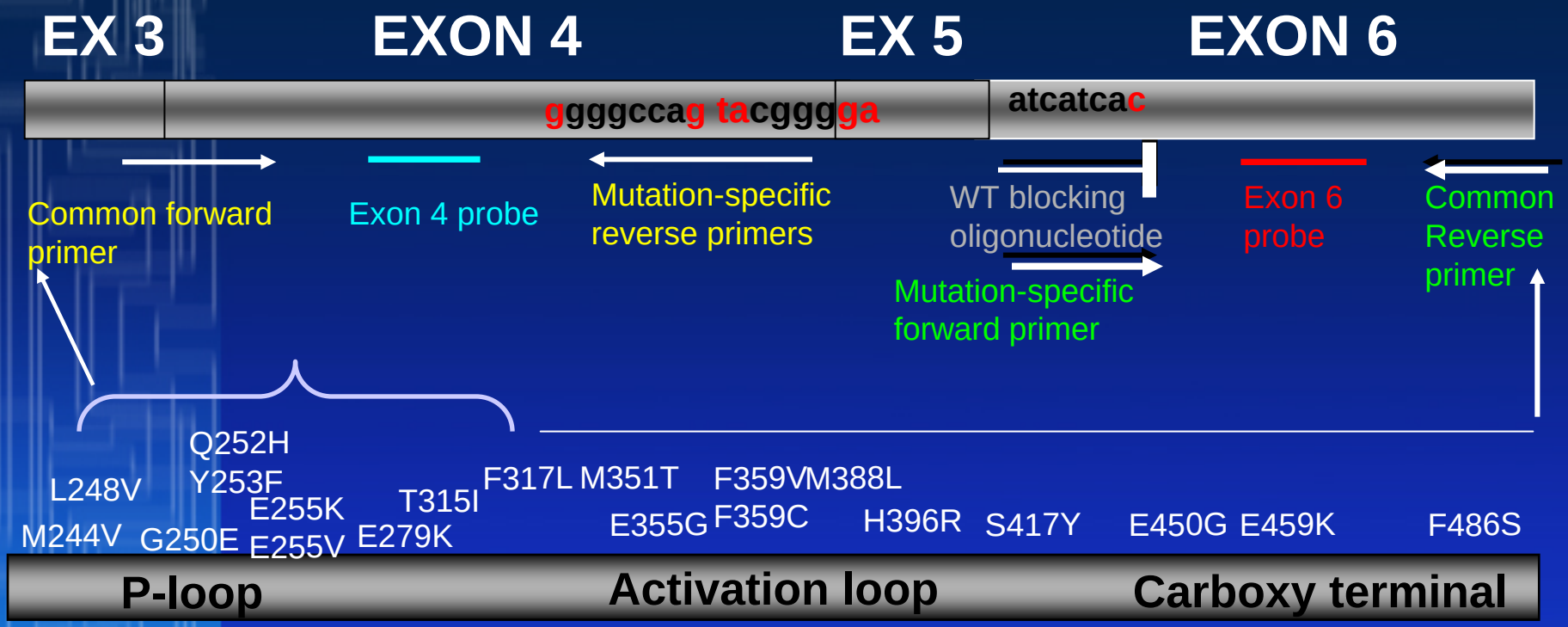

Diagram representing the spectrum of Abl tyrosine kinase domain mutations and a magnification view of the specific mutations in exons 4 and 6 identified by our PCR assays.

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Experimental methods

- **Model Systems**
  - Bcr-Abl plasmids for all mutations and wild-type generated from K562 cell line by site directed mutagenesis
  - Bcr-Abl cDNA for wild-type, T315I, and Y253F mutations generated from BaF3 cells
  - Mutation-specific primers had 2 mismatches relative to the WT sequence and 1 mismatch relative to the mutant sequence
  - Black Hole Quencher probes specific to exon 4 (p-loop) and to exon 6 (T315I)

- **Clinical samples**
  - total random-primed cDNA was prepared from RNA extracted from Ficoll-separated peripheral blood
  - Total Abl was also amplified using primers that would detect both mutated and WT cDNA
  - The housekeeping gene beta-2 microglobulin was amplified to assess RNA integrity

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Data from Model Mixtures

Single Q252H G>C and G>T Mutant Plasmid Copies Detected in Background of up to 10^5 WT Bcr-Abl cDNA Copies

Q252H G>C Cycle 32 Q252H G>T

67 copies mut in 50,000 copies WT

64 copies mut only

64 copies mut in 100,000 copies WT

100,000 copies WT only

125 copies mut in 50,000 copies WT

26 copies mut only

29 copies mut in 100,000 copies WT

100,000 copies WT only

CAL-Fluor Orange fluorescent image of the Digital Array after 32 cycles of mutation-specific PCR for the Q252H G>C and Q252H G>T mutations. Actual mutant and WT copy numbers in the standard dilutions are shown. Other mutation-specific PCR assays were developed for the following mutations: G250E, Y253F, E255K, E255V, and T315I.

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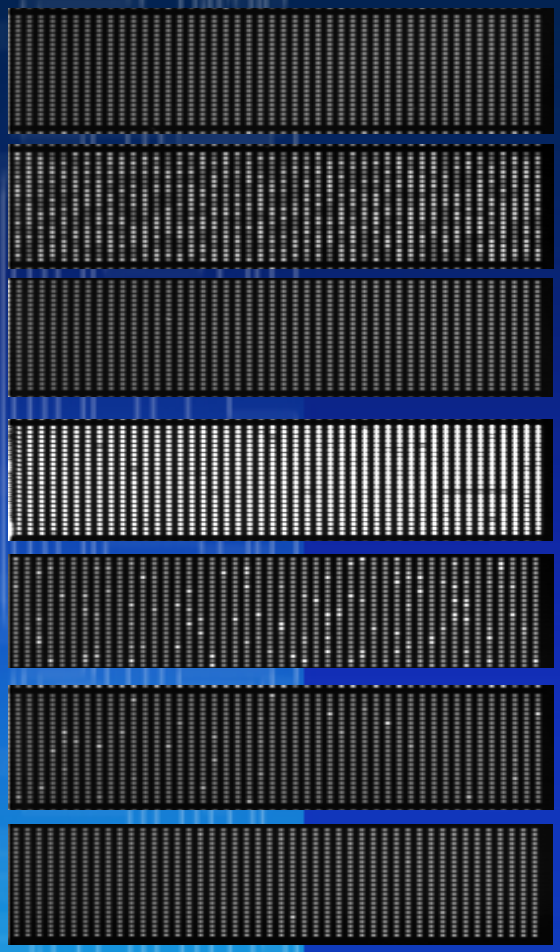
Digital Array Chip Improves the Detection Level of the T315I Abl Tyrosine Kinase Domain Mutation in cDNA from CML Patients

T315I-specific Cycle 39 **Total Abl**



No cDNA Control

Positive Control: Mutant T315I BCR-Abl cDNA from BaF3

Negative Control: Non-CML Patient PBL (0 positives)

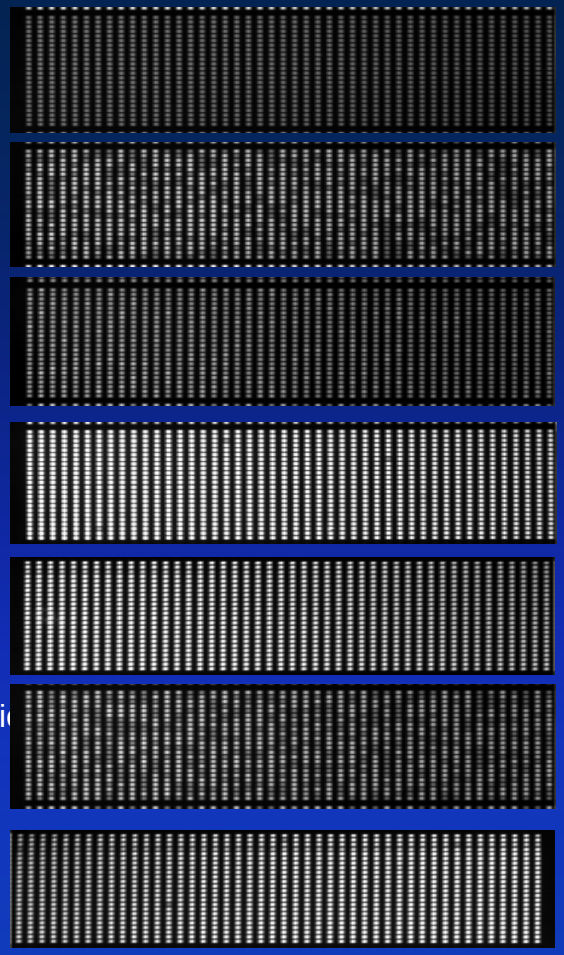
Sample: IM Resistant Patient 4 (1100+ positives)

Sample: IM Resistant Patient 10 (99 positives)

Sample: IM Resistant Pati 1 (31 positives)

Sample: IM Resistant Patient 10, Early Sample

(1 positive)



No cDNA Control

Positive Control: Mutant T315I BCR-Abl cDNA from BaF3 Cell Line

Negative Control: Non-CML Patient PBL (314 positives – amplifying normal Abl)

Sample: IM Resistant Patient 4 (1100+ positives)

Sample: IM Resistant Patient 10 (1100+ positives)

Sample: IM Resistant Patient 1 (~660 positives)

Sample: IM Resistant Patient 10, Early Sample (1100+ positives)

A) T315I mutant-specific PCR on the Digital Array is able to detect and quantify single mutant copies in the background of at least 10^3 copies of Abl.

B) Total Abl was amplified by PCR with primers exterior to codon 315 on the Digital array. A saturated signal occurs with more than 10^3 copies. Further sample dilutions are required for absolute quantification of total Abl above these levels.

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Results

- Model systems using serial dilutions of plasmid constructs detected mutant copies levels in wild type background ranging from 1:1000 to 1:10,000
- Cell lines controls demonstrated similar sensitivity as the plasmid models.
- For the most common mutation T315I, clinical samples of three patients showed sensitivity of 1:1000 versus 1:4 by sequencing method
- The sensitivity improvement should be useful in clinical settings. Further studies should explore increased number of sample chambers and establish the statistical limit of detection with real-time PCR

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Challenges

- Protein Crystallography
  - Eliminate scale up and detect directly in the beam
  - Automated reagent formulation
  - New materials for incompatible reagents
- Digital Array
  - Single molecule detection without amplification
  - Protein detection
- Devices
  - Increase density
  - Lower cost of manufacture
  - Control logic
  - Integrated detection

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The Promise of Complex Microfluidic Circuitry

- Integration improves reliability and enables large studies
- Scale reduces reagent costs
- Novel physics enables new experiments

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Further information

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