Polony Technology for Sequencing and Genotyping

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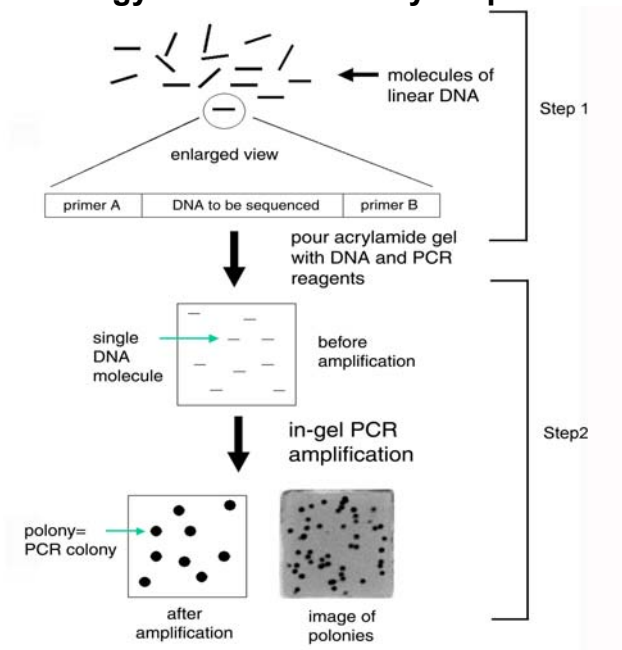
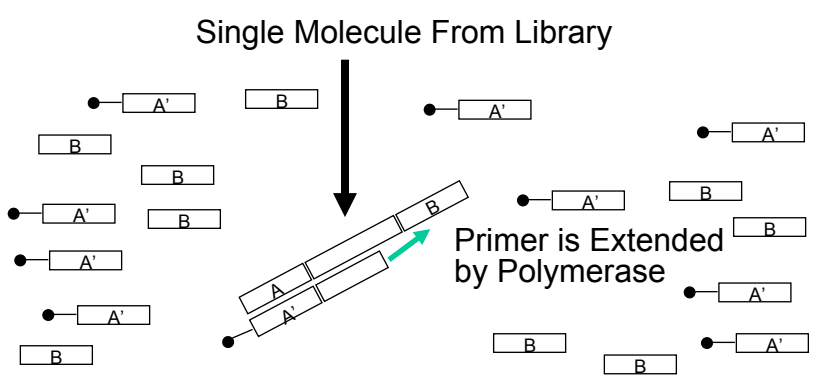
High Throughput Sequencing Applications

- De novo sequencing
- Resequencing (SNP discovery)
- SNP scoring
- Whole-genome mRNA abundance measurements
- Readout for genetic selections or screens

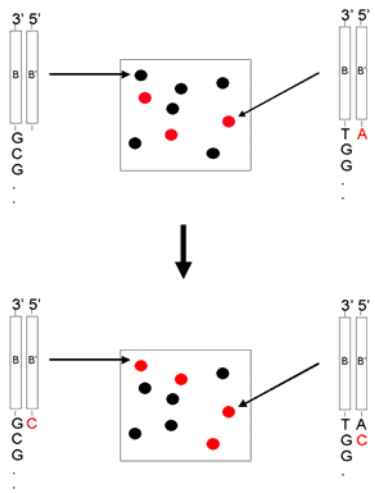
Technology Overview: Polony Amplification



Primer A has 5' Acrydite Modification



Fluorescent In Situ Sequencing



Base Extension with dATP

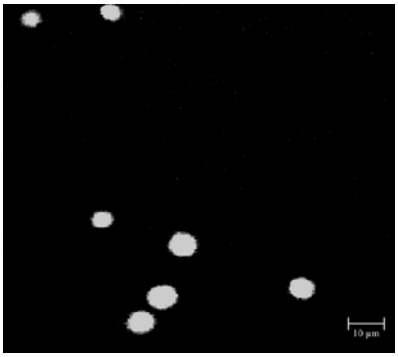
Remove Fluorescence

Base Extension with dCTP

Advantages of Polony Sequencing

- Miniaturization (subpicoliter volumes)
- Parallel nature
- Integrated sample preparation
- Goal: 500 million bases per slide

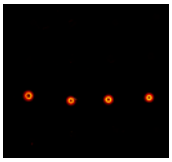
Polony Radius can be as small as 5 microns



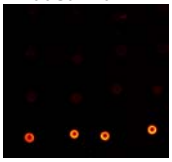
At this density, more than 5 million distinguishable polonies per slide

Single Base Extensions on Gel-Immobilized Oligonucleotide Templates

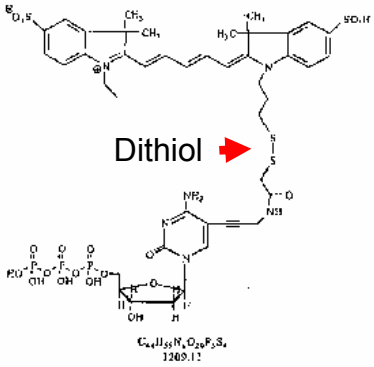
Next Base	Added fl-dUTP	Fluorescent Intensity
T		100 +/- 6.0
G		0.59 +/- 0.5
C		0.58 +/- 0.4
A		0.18 +/- 0.2

Next Base	Added fl-dCTP	Fluorescent Intensity
T		0.55 +/- 0.1
G		0.42 +/- 0.1
C		100 +/- 5.3
A		0.30 +/- 0.1

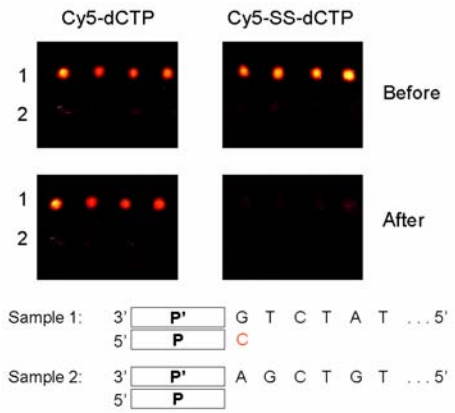
	Added fl-dGTP	
T		1.5 +/- 1.0
G		100 +/- 6.2
C		0.8 +/- 0.3
A		2.4+/- 0.4

	Added fl-dATP	
T		3.4 +/- 0.5
G		5.6 +/- 0.8
C		8.8 +/- 0.8
A		100 +/- 10.8

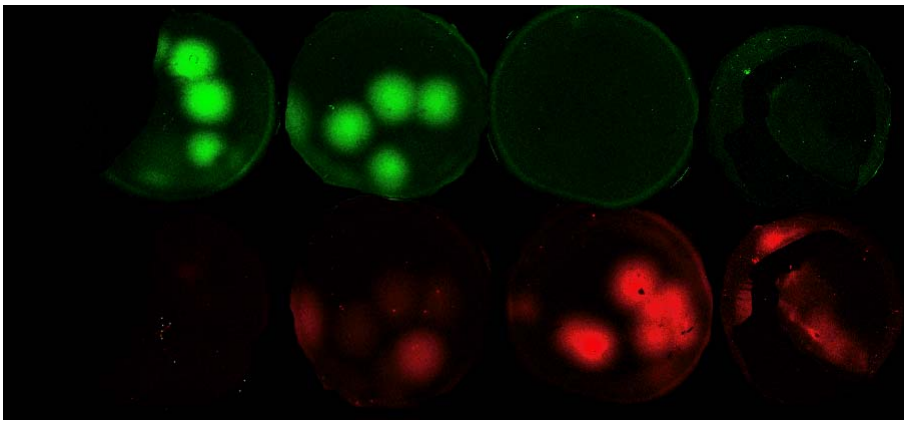
Incorporated Fluorescence Can be Removed Using a Modified Nucleotide



Cy 5-SS-dCTP



Reaction Efficiency = 99.8%



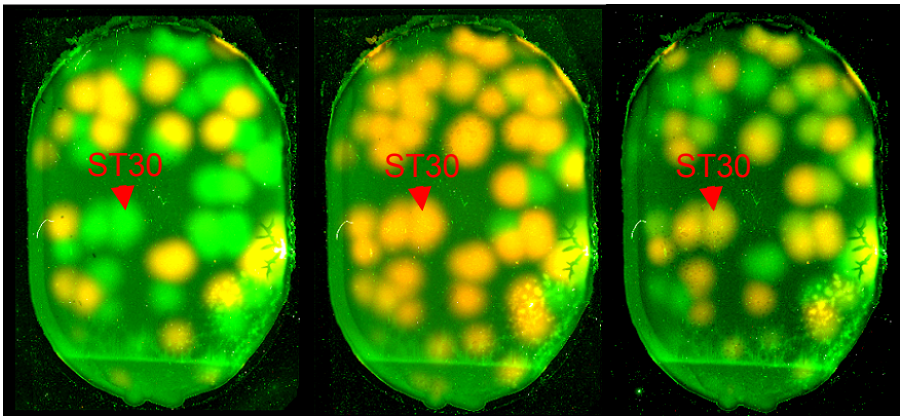
Mean Intensity: 58, 0.5 40, 6.5 0.3, 48 0.4, 43

Polony Template

3' P' TATTGTTAAAGTGTGTCCTTTGTCGATACTGGTA...5'
5' P ATAACAATTTCACACAGGAAACAGCTATGACCAT

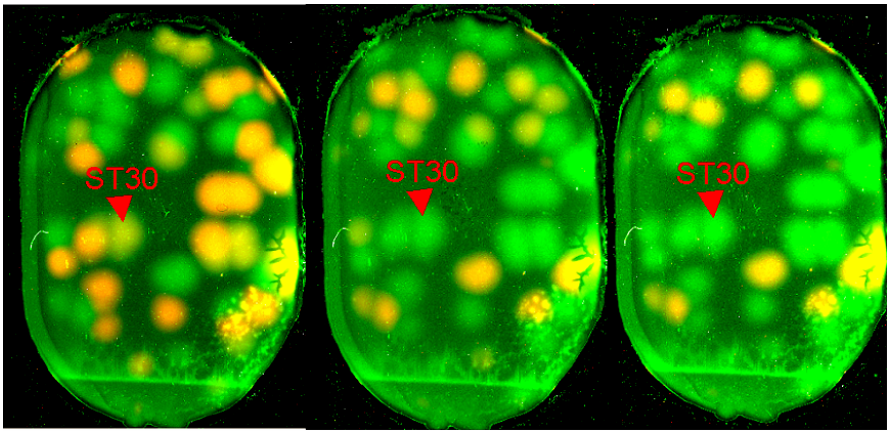
FITC (C) **CY3 (T)**

Sequencing on Polonies



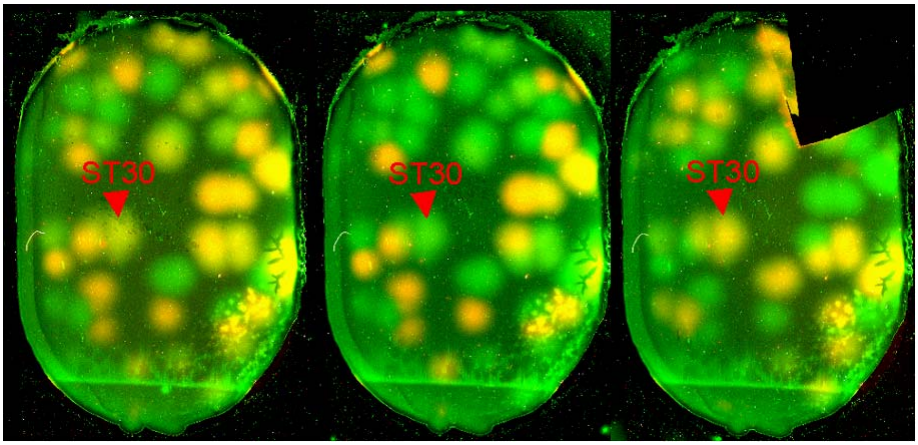
	1	2	3
ST30 3'	P' TCACGAGT	P' TCACGAGT	P' TCACGAGT
5'	P	P A	P AG
Base Added:	C	A	G

Sequencing on Polonies



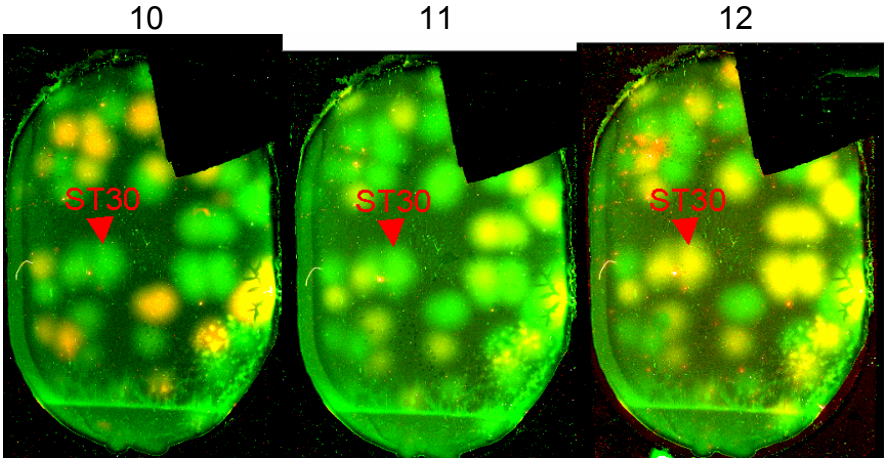
	4	5	6
ST30 3'	P' TCACGAGT	P' TCACGAGT	P' TCACGAGT
5'	P AGT	P AGT	P AGT
Base Added:	T	C	A

Sequencing on Polonies



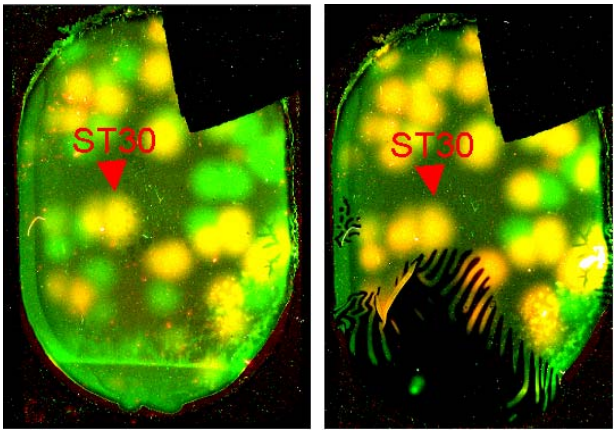
	7	8	9
ST30 3'	P' TCACGAGT	P' TCACGAGT	P' TCACGAGT
5'	P AGTG	P AGTG	P AGTGC
Base Added:	G	T	C

Sequencing on Polonies



	10	11	12
ST30 3'	P' TCACGAGT	P' TCACGAGT	P' TCACGAGT
5'	P AGTGC	P AGTGC	P AGTGCT
Base Added:	A	G	T

Sequencing on Polonies



ST30
3' P' TCACGAGT
5' P AGTGCTC

ST30
3' P' TCACGAGT
5' P AGTGCTCA

Base Added: C A

Remaining Problems

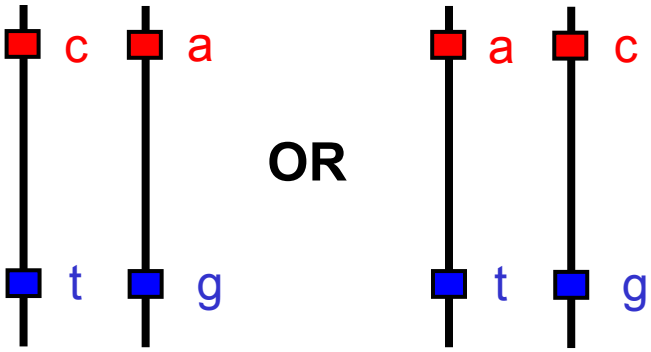
- Gel attachment
- Calling multiple base runs (context dependence of fluorophore quantum yield)
- Cycle time
- Increase sequencing accuracy

Other Applications of Polonies

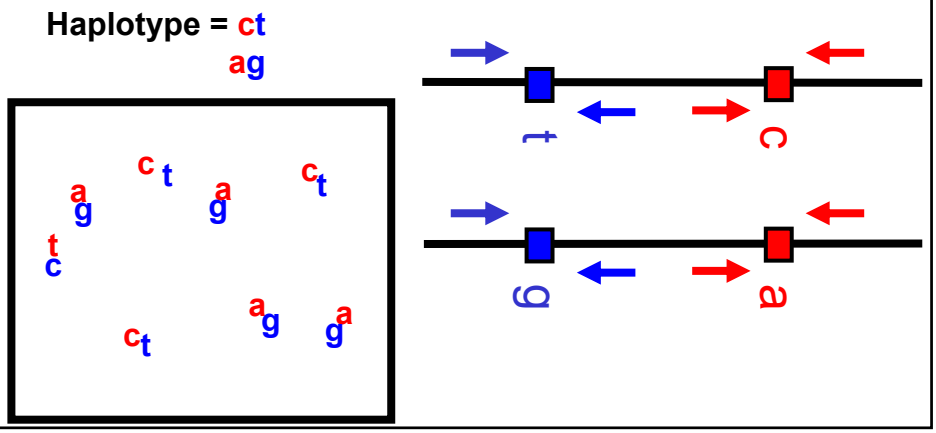
- Genotyping single molecules
  - LOH analysis
  - Detecting rare SNPs (1 in 10^3)
- Haplotyping
- Exon Analysis

Direct Molecular Haplotyping with Polonies

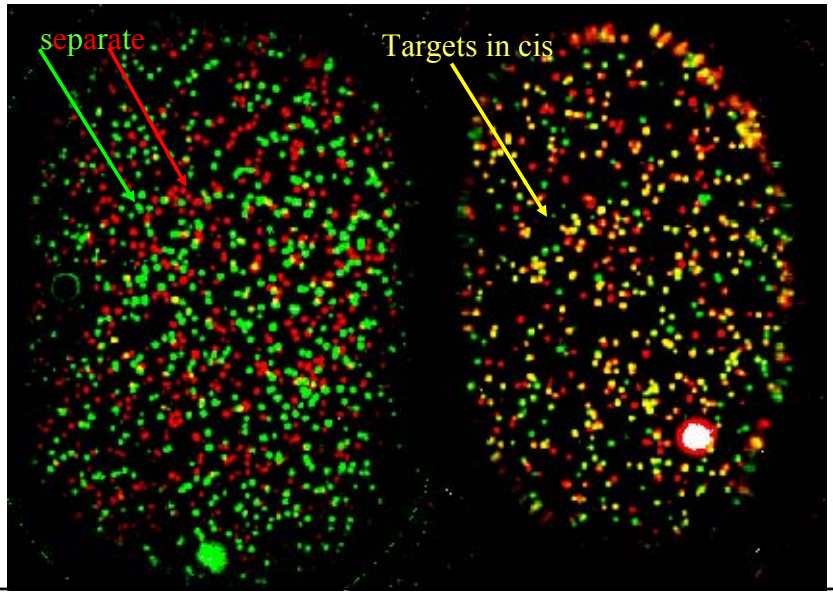
- Determine phasing of SNPs over very long distances: Genotype (c/a t/g)



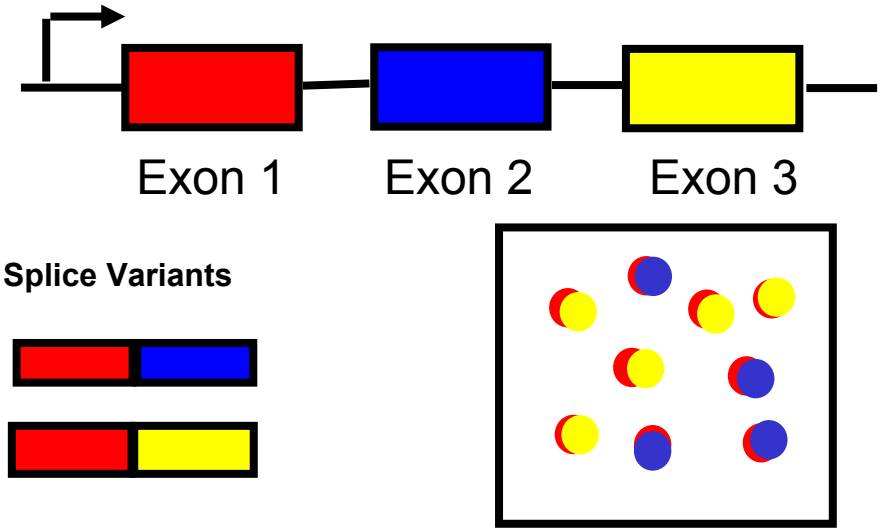
Coamplify Two or More Loci and Genotype



Single molecule haplotypes (or RNA) detection: in situ amplified "polonies" (85% efficiency)



Exon Analysis



Conclusions

- A large number of DNA template molecules can be amplified and manipulated in parallel using polony technology
- The amplified DNA can be extended by multiple rounds of nucleotide addition to give sequence information
- Polony technology may prove useful for genotyping, haplotyping, and exon typing

Acknowledgements

George Church

Vincent Butty

Phil Busby

Aimee Dudley

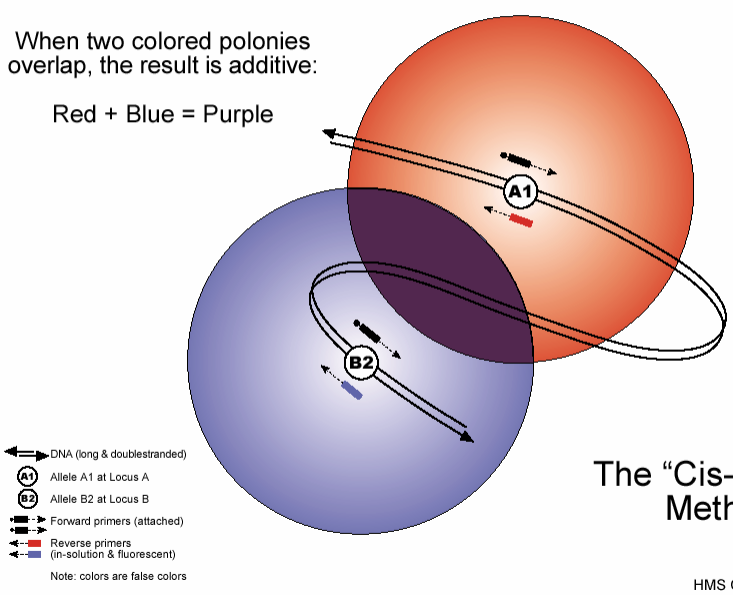
The Church Lab

We are poster number 47,
please stop by to see data from other projects

Multiple polony DNA polymorphism linkage analysis

When two colored polonies overlap, the result is additive:

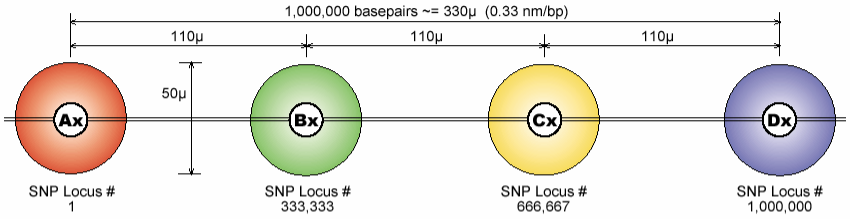
Red + Blue = Purple



The "Cis-Polony" Method

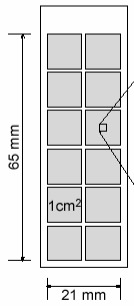
HMS Case 1929, Fig. 5

1 Mb cis-polony DNA polymorphism linkage analysis



Slide area not bearing ink-jetted primers is:

- wettable if DNA is from one patient
- non-wettable if DNA is from multiple patients



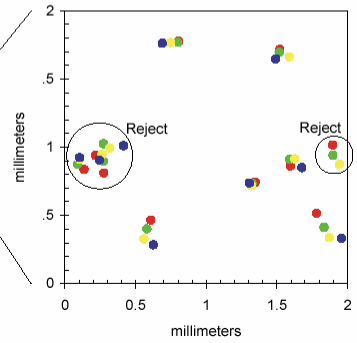
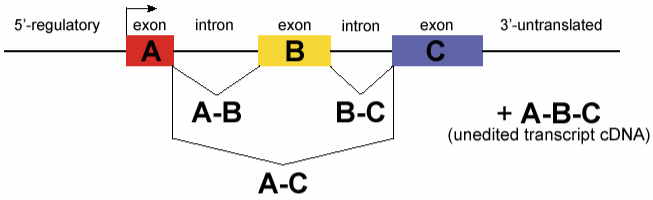


Image Informatics

1. Reject any similarly colored polonies closer together than 1/2 fully extended molecule length (eg, 165μ ctr-to-ctr)
2. Reject any polony clusters that do not have exactly 4 members:
   - <4 = shear-damaged DNA or PCR artefact
   - >4 = random co-localization of template DNA molecules
3. Reject any clusters where polonies are not within predictable distances of each other

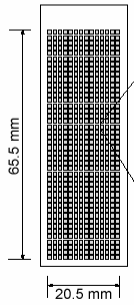
HMS Case 1929, Fig. 6

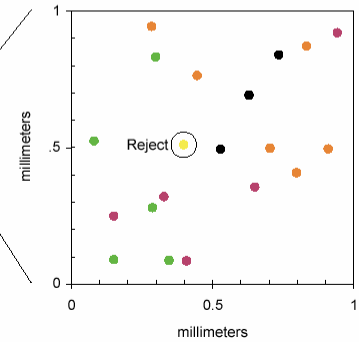
Cis-polony mRNA "exon-typing" analysis



Slide area not bearing ink-jetted primers is:

- wettable if DNA is from one patient
- non-wettable if DNA is from multiple patients
- 616 features/slide each 1mm2





Reject any polony clusters that have only one member

Results:

01 B	(yellow) reject	
06 A-B	(orange)	32%
05 B-C	(green)	26%
05 A-C	(purple)	26%
03 A-B-C	(black)	16%
19 accepted		100%

HMS Case 1929, Fig. 17

Polony Sequencing for SNP screening

Patient

↓

Swab buccal cells from mouth

↓

Create *in vitro* genomic library

↓

Enrich library using Hybrigel technology

↓

Perform pSeq

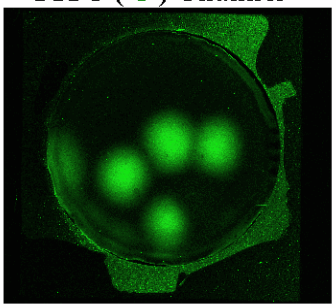
↓

1.5 million tag sequences
100,000 SNP (P=99.9%)

15 base additions, 22 incorporation events

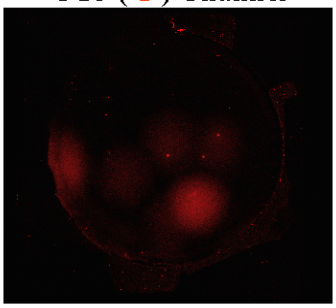
CYCLE:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
BASE:	A	T	A	C	A	T	G	A	C	A	C	A	G	A	C / T

FITC (C) Channel



Mean Polony Intensity: 39.5

CY3 (T) Channel



Mean Polony Intensity: 6.5

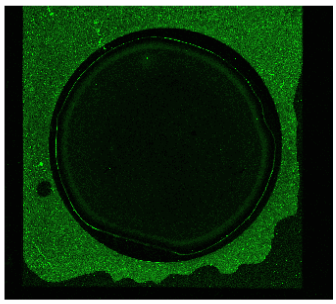
Polony Template

3' [P'] T A T T G T T A A A G T G T G T C C T T T G T C G A T A C T G G T A ...5'
5' [P] A T A A C A A T T T C A C A C A G G A A A C

19 base additions, 26 incorporation events

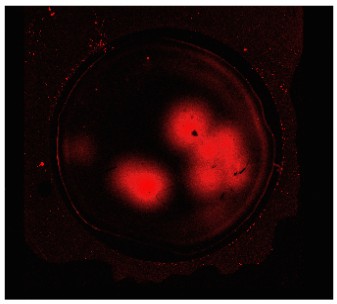
CYCLE:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
BASE:	A	T	A	C	A	T	G	A	C	A	C	A	G	A	C	A	G	C	C / T

FITC (C) Channel



Mean Polony Intensity: 0.25

CY3 (T) Channel



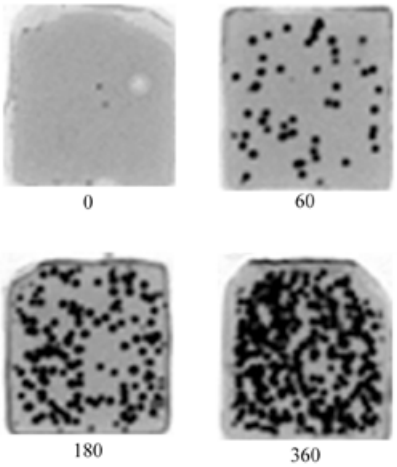
Mean Polony Intensity: 48.2

Polony Template

3' [P'] T A T T G T T A A A G T G T G T C C T T T G T C G A T A C T G G T A ...5'

5' [P] A T A A C A A T T T C A C A C A G G A A A C A G C T

Polony Amplification



Efficiency of Extension Reaction

Error accumulates exponentially with
the number of incorporated bases

Assume reaction goes to 85% completion

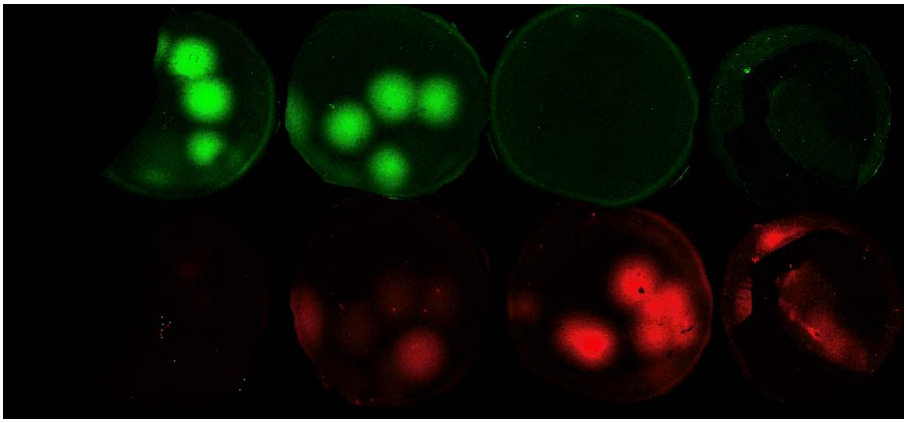
After 6 extensions,
(85%)^6 = 38% of the template molecules are extended correctly

The rest of the molecules are “out of phase”

Future Direction

- Demonstrate sequencing out to 20 bases and determine error rate
- Automate sequencing protocol
- Demonstrate sequencing on polonies < 20 microns
- Demonstrate ability to haplotype alleles > 20kb apart

Primer Extension 26 cycles, 34 Nucleotides



Mean Intensity: 58, 0.5 40, 6.5 0.3, 48 0.4, 43

Polony Template

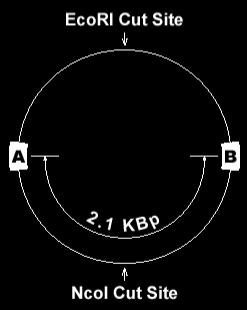
3' P' TATTGTTAAAGTGTGTCCTTTGTCGATACTGGTA…5'
5' P ATAACAATTTCACACAGGAAACAGCTATGACCAT

FITC (C) **CY3 (T)**

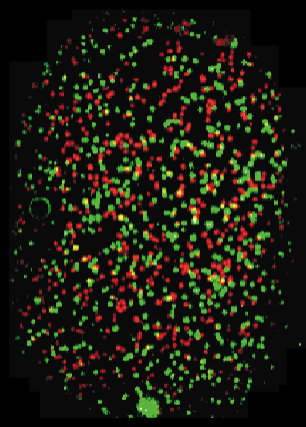
Proof of concept experiment

- Locus A polony (85% yield)
- Locus B polony (81% yield)
- Loci A+B cis-polony (69%)

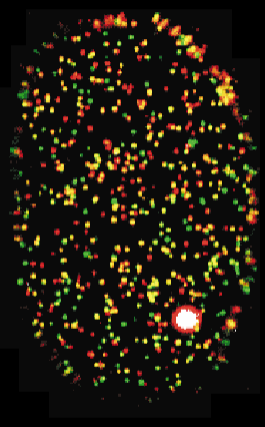
(polonies are ~300 μ diam)



Plasmid DNA Template
(not drawn to scale)



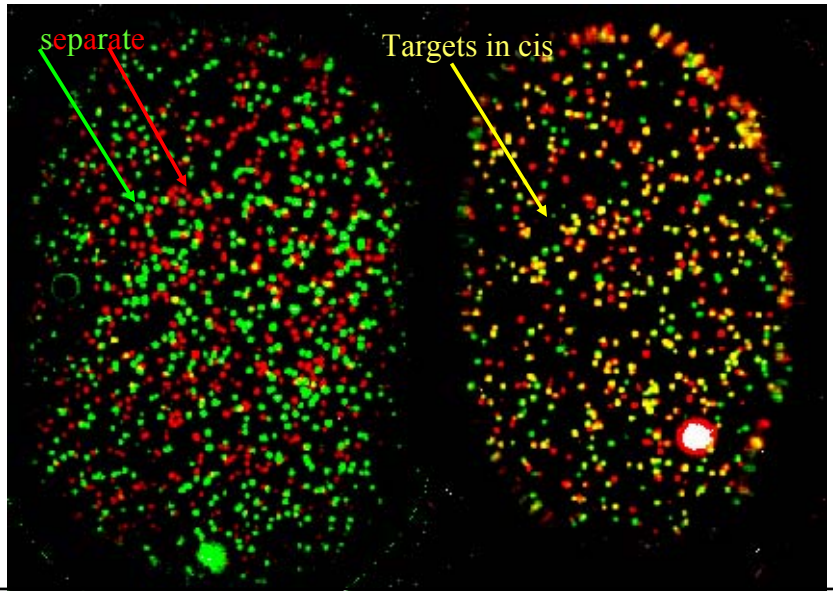
Double Cut
(EcoRI & NcoI)



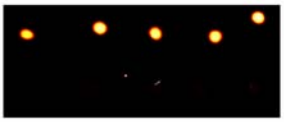
Single Cut
(EcoRI only)

HMS Case 1929, Fig. 3

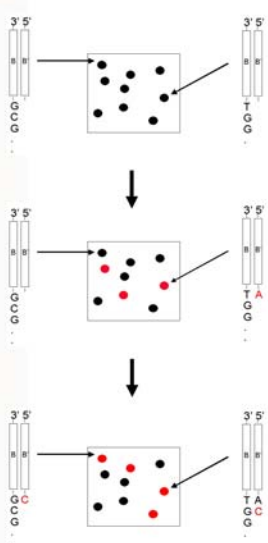
Single molecule haplotypes (or RNA) detection: in situ amplified "polonies" (85% efficiency)



Extension estimated to go to 99.8% completion

Extension with unlabeled dTTP	Next Base	Polymerase Trapping	Fluorescent Intensity
0 seconds	T		100 +/- 14.5
	C		1.83 +/- 0.024
60 seconds	T		1.97 +/- 1.38
	C		1.73 +/- 1.22

Technology Overview: Polony Sequencing



1. Denature DNA
2. Anneal universal sequencing primer
3. Add DNA polymerase and fluorescently labeled dATP
4. Wash
5. Scan gel
6. Remove fluorescent label from incorporated base
7. Add fluorescently labeled dCTP
8. Wash
9. Scan gel

Step 3

High Throughput Sequencing

Millions of DNA fragments

↓

Clone

↓

Amplify

↓

Sequence

↓

Processed