Next-Generation Sequencing Technologies

Elaine R. Mardis
Professor in Genetics
Co-director, The Genome Institute

NHGRI Current Topics in Genome Analysis

Current Topics in Genome Analysis 2012

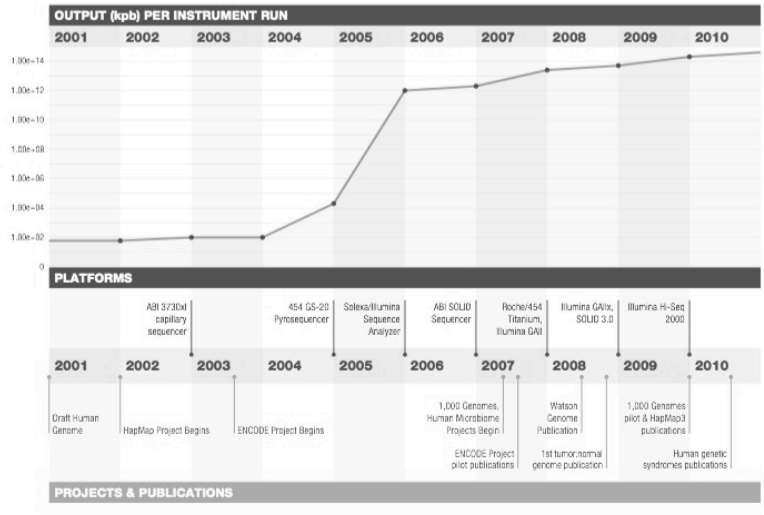
Elaine Mardis, Ph.D.

No Relevant Financial Relationships with Commercial Interests

Overview

- Next-Generation Sequencing (NGS) Instruments
  - Roche/454
  - Illumina
  - Life Technologies
  - Pacific Biosciences
  - Ion Torrent
  - Oxford Nanopore
- NGS Applications across the spectrum of genomics
  - Examples from our work
  - Future Directions

The Trajectory of Throughput: 10 years



E.R. Mardis, Nature (2011) 470: 198-203

Comparative costs: sequencing a human genome

Capillary technology

Applied Biosystems 3730xl (2004)

$15,000,000

Next-gen technology

Illumina HiSeq (2011)

$10,000

Next-generation Sequencer basics

Platforms and their attributes

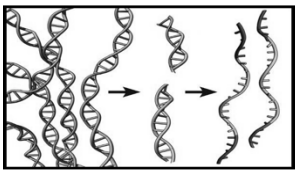
Next-generation DNA sequencing instruments

- All commercially-available sequencers have the following shared attributes:
  - Random fragmentation of starting DNA, ligation with custom linkers = "a library"
  - Library amplification on a solid surface (either bead or glass)
  - Direct step-by-step detection of each nucleotide base incorporated during the sequencing reaction
  - Hundreds of thousands to hundreds of millions of reactions imaged per instrument run = "massively parallel sequencing"
  - Shorter read lengths than capillary sequencers
  - A "digital" read type that enables direct quantitative comparisons
  - A sequencing mechanism that samples both ends of every fragment sequenced ("paired end" reads)

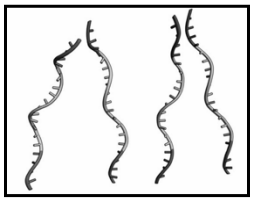
Paired-end reads

- All next-gen platforms now offer paired end read capability, e.g. sequences can be derived from both ends of the library fragments.
- Differences exist in the _distance_ between read pairs, based on the approach/platform.
  - "paired ends" : linear fragment sequenced at both ends in two separate reactions
  - "mate pairs" : circularized fragment of >1kb, sequenced by a single reaction read or by two separate end reads (platform dependent)
- In general, paired end reads offer advantages for sequencing large and complex genomes because they can be more accurately placed ("mapped") than can single ended short reads.

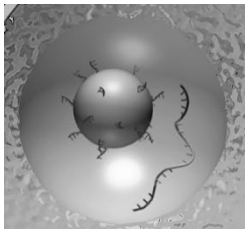
Roche/454 Library Construction and emPCR



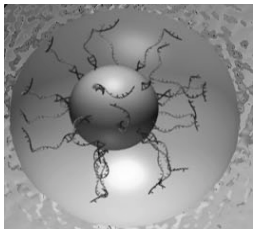
•Random Fragmentation
•Adapter Ligation



Single Stranded Adapter Ligated Library

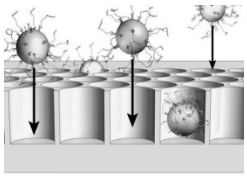


• emPCR for clonal amplification on bead



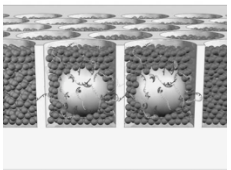
Roche/454 Pyrosequencing

Load beads into PicoTiter™Plate



Load Enzyme Beads

Centrifugation



DNA Capture Bead Containing Millions of Copies of a Single Clonal Fragment

Sequencing by Synthesis

A A T C G G C A T G C T A A A A G T C A

T

Anneal Primer

Sulfurylase

Luciferase

APS

PP_i

ATP

luciferin

Light + oxy luciferin

454 Instrumentation

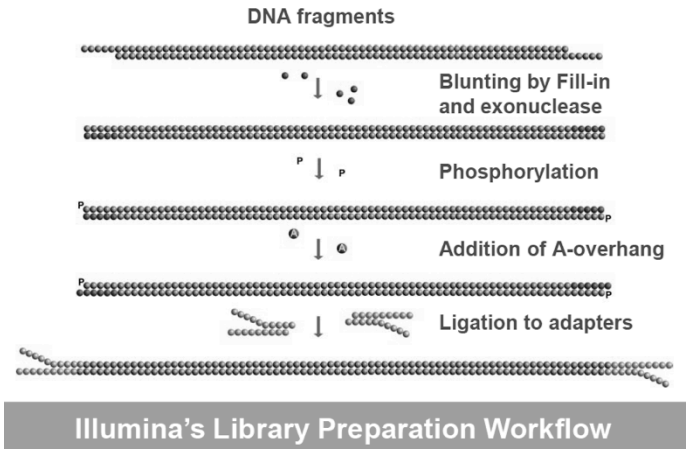
Instrument	Run Time (hr)	Read Length (bp)	Yield (Mb/run)	Error Type	Error Rate (%)	Purchase Cost (x1000)
454 FLX+	18-20	700	900	Indel	1	$30 [A]
454 FLX Titanium	10	400	500	Indel	1	$500
454 GS Jr. Titanium	10	400	50	Indel	1	$108

[A] – Requires the 454 FLX Titanium. This is the upgrade cost.

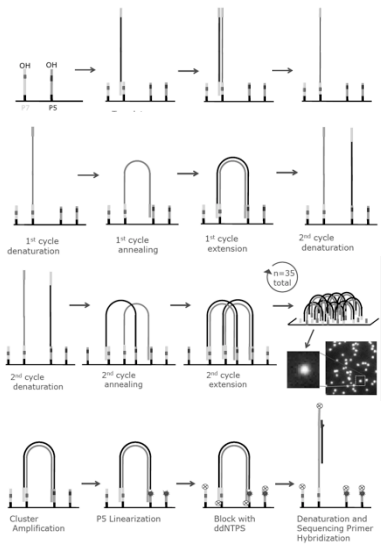
Notable:

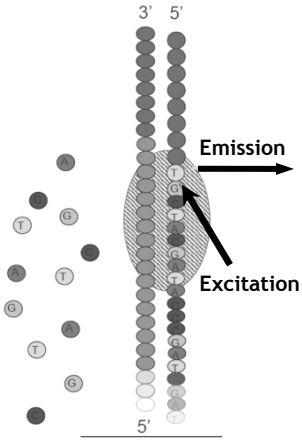
- Mate pair paired end reads of 3kb, 8kb and 20 kb separation without an increase in run time.
- Cost per run makes sequencing an entire human genome cost-prohibitive relative to other technologies (~ $20/Mbp)
- Great platform for targeted validation

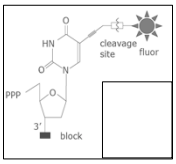
Illumina Sequencing: Library Preparation



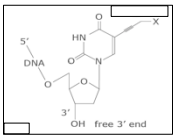
Illumina Sequencing by Synthesis







Incorporate
Detect
De-block
Cleave fluor

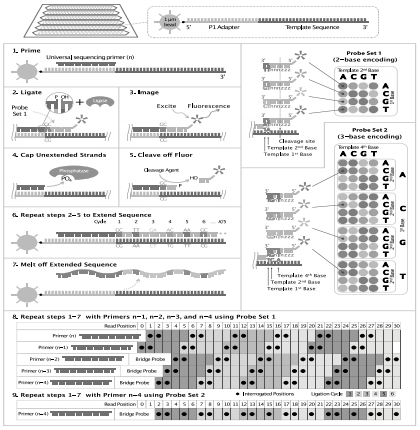


Illumina Instrumentation

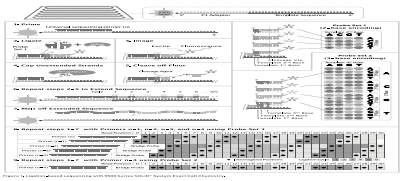
- 2010: HiSeq 2000
  - Two flow cells per run
  - 100 Gbp/FC or two genome equivalents per run
  - New scanning mechanics - scans both surfaces of FC lanes
- 2011: HiSeq 2000
  - Improved chemistry (v. 3): increased yield and accuracy
- 2011: MiSeq

Instrument	Run Time (days)	Read Length (bp)	Yield (Gb/run)	Error Type	Error Rate (%)	Purchase Cost (x1000)
GAIIx	14	150 x 150	96	Sub	>0.1	$525
HiSeq 2000	8	100 x 100	200 x 2	Sub	>0.1	$700
HiSeq 2000 v3	10	100 x 100	<600	Sub	>0.1	$700
MiSeq	1	150 x 150	2	Sub	>0.1	$125

Life Technologies: sequencing by ligation



- custom adapter library
- emPCR on magnetic beads
- sequencing by ligation using fluorescent probes from a common primer
- sequential rounds of ligation from a series of primers
- fixed/known nucleotides for each probeset identify two bases each cycle, or "two base encoding"



SOLiD Instrumentation

Instrument	Run Time (days)	Read Length (bp)	Yield (Gb/run)	Error Type	Error Rate (%)	Purchase Cost (x1000)
SOLiD 4	12	50 x 35 PE	71	A-T Bias	>0.06	$475
SOLiD 5500 xl	8	75 x 35 PE 60 x 60 MP	155	A-T Bias	>0.01	$595

5500 xl

- Front-end automation addresses bottlenecks at emPCR, breaking, and enrichment of beads
- 6-lane Flow Chip with independent lanes/2 per run
- Cost per whole genome data set is predicted to be $6K by 2011
- Very high accuracy data due to two-base encoding
- ECC Module – An optional 6th primer that increases accuracy to 99.999%
- Direct conversion of color space to base space
- True paired-end chemistry enabled – Ligation reaction can be used in either direction

Third generation sequencers??

- Recently, new sequencing platforms were introduced.
- The Pacific Biosciences sequencer is a single molecule detection system that marries nanotechnology with molecular biology.
- The Ion Torrent uses pH rather than light to detect nucleotide incorporations.
- The MiSeq is a scaled down version of the HiSeq, with faster chemistry and scanning.
- All offer a faster run time, lower cost per run, reduced amount of data generated relative to 2nd Gen platforms, and the potential to address genetic questions in the clinical setting.

Comparisons to Third-Generation Sequencers

Company	Platform Name	Sequencing	Amplification	Run Time
Roche	454 Ti	DNA Polymerase "Pyrosequencing"	emPCR	10 hours
Illumina	Hi-Seq/ MiSeq	DNA Polymerase	Bridge amplification	10 days/ 24 hours
Life	SOLiD/ 5500	DNA Ligase	emPCR	12 days
Ion Torrent	PGM	Synthesis H^+ detection	emPCR	2 hours
Pacific Biosciences	RS	Synthesis	NONE	45 min

Pacific Biosciences RS

Sample Prep

Shearing (Covaris/Hydroshear)
↓
Polish ends
↓
SMRTbell™ ligation
↓
Sequencing primer annealing

Library/Polymerase Complex

DNA polymerase binding
↓
Load library/polymerase complex onto SMRT cell
↓

Sequencing

Movie 1 (v1.1 & v1.2)
Raw reads → Post-filter reads → Mapped reads

Movie 2 (only v1.2)
Raw reads → Post-filter reads → Mapped reads

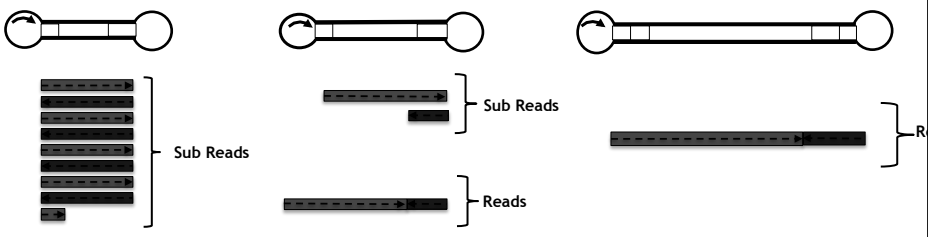
Zero Mode Waveguides (ZMWs)
Ver. 1.1 = 1 x 45,000 per SMRT cell
Ver. 1.2 = 2 x 75,000 per SMRT cell

The SMRTbell™ Library Types

Circular Consensus
Small ~250bp
Single Movie
Multiple passes
Many sub-reads

Standard
Large ~2kbp
Single Movie
Few sub-reads

VLR ("very long read")
Larger ~6kbp
Single 45 minute Movie
Single long read provides linking information

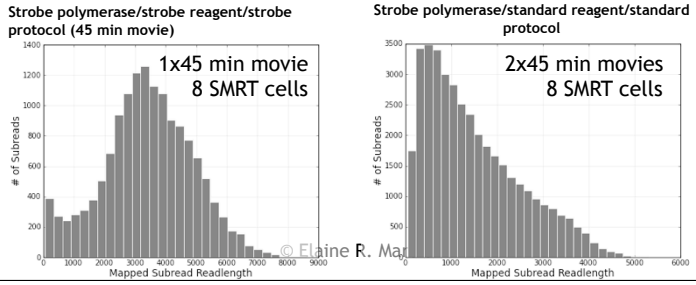


Consensus Read

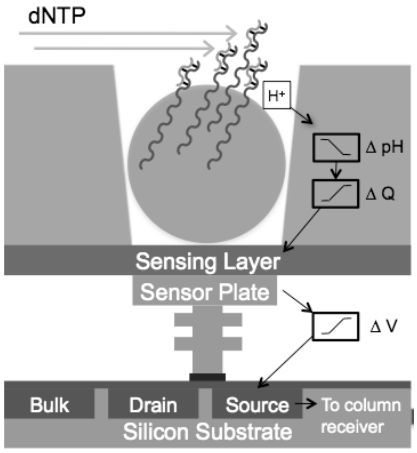
PacBio RS Instrumentation

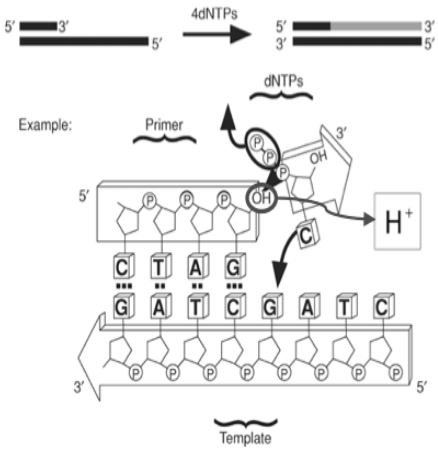
Instrument	Run Time (Hours)	Read Length (bp)	Yield (Mb)	Error Type	Error Rate (%)	Purchase Cost (x1000)
RS	14 (~8 SMRTCells)	2500	45 per SMRTCell	Insertions	15	$695

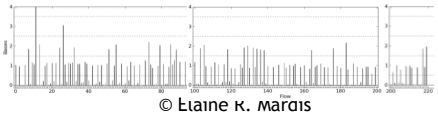
mean mapped sub-read accuracy: 86.2%
mean mapped sub-read length: 3,416 bp
maximum mapped read length: 8,580 bp
maximum 95th percentile mapped read length: 5,807 bp



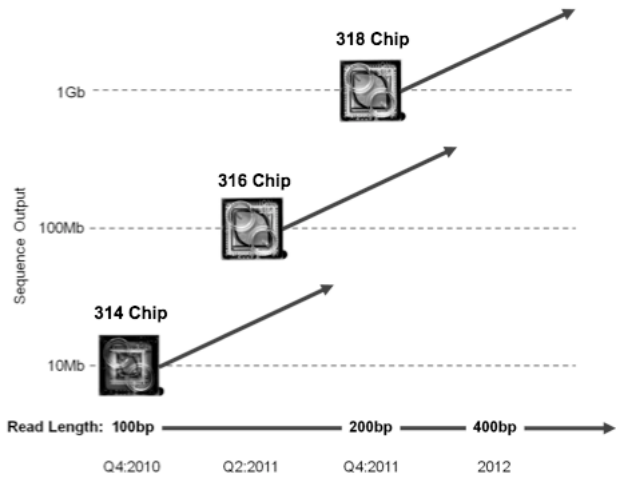
ION Torrent Personal Genome Machine (PGM)



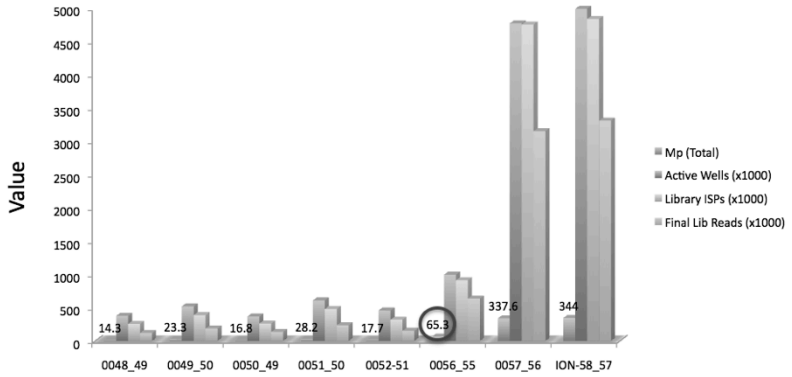




Ion Torrent Yield Trajectory



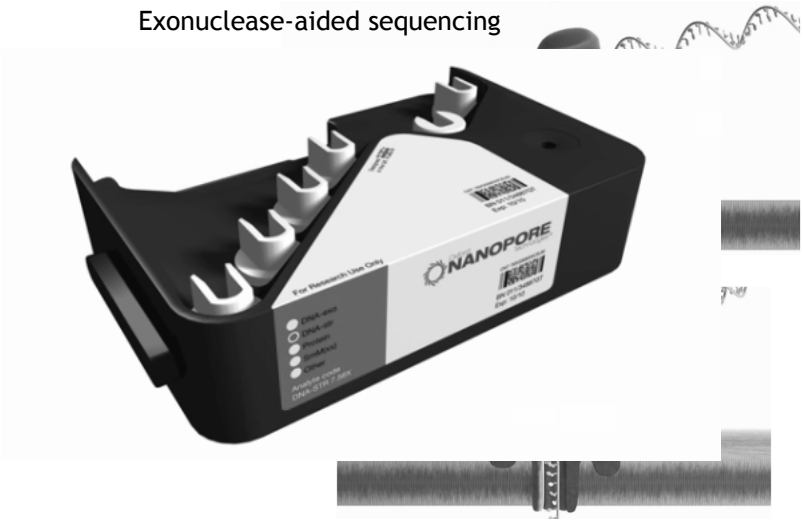
Ion Torrent Yield with Automation Modules



	0048_49	0049_50	0050_49	0051_50	0052-51	0056_55	0057_56	ION-58_57
Chip	**314**	**314**	**314**	**314**	**314**	**314**	**316**	**316**
Sample	***Ef***	***Ef***	***Ef***	***Ef***	***Ef***	***Ec***	***Ec***	***Ec***
Emulsion	**Man**	**Man**	**Man**	**Man**	**Man**	**1-T**	**1-T**	**1-T**
Enrichment	**Man**	**Man**	**Man**	**Man**	**Man**	**ES**	**ES**	**ES**

Oxford Nanopore Sequencing

Exonuclease-aided sequencing



Applying Next Generation Sequencing

- Genomes: re-sequencing or *de novo*
- point mutation/indel/structural variation discovery
- Protein:DNA binding
  - Chromatin IP/histone binding
  - Nucleosome/transcription factor binding, etc.
- ncRNA discovery/sequencing/variants
- Transcriptome sequencing (RNA-seq)
- Genome-wide methylation of DNA (Methyl-seq)
- Clinical sequencing for therapeutic decisions

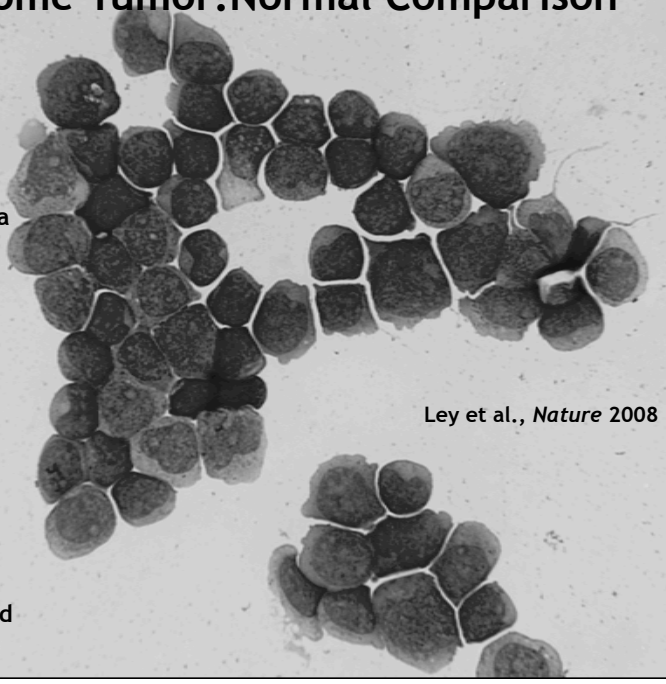
E.R. Mardis, Annual Reviews in Genetics & Genomics (2008)
E.R. Mardis, Nature (2011) 470: 198-203

Whole Genome Sequencing: Data Production and Alignment

- Prepare paired end libraries as whole genome fragment/shotgun by random shearing of genomic DNA, adapter ligation, size selection.
- Produce paired end data from each end of billions of library fragments, over-sampling about 30-fold to cover at a depth sufficient to find all types of genome alterations.
- Computer programs align the read pair sequences onto the reference genome and several algorithms are used to discover variants genome-wide.

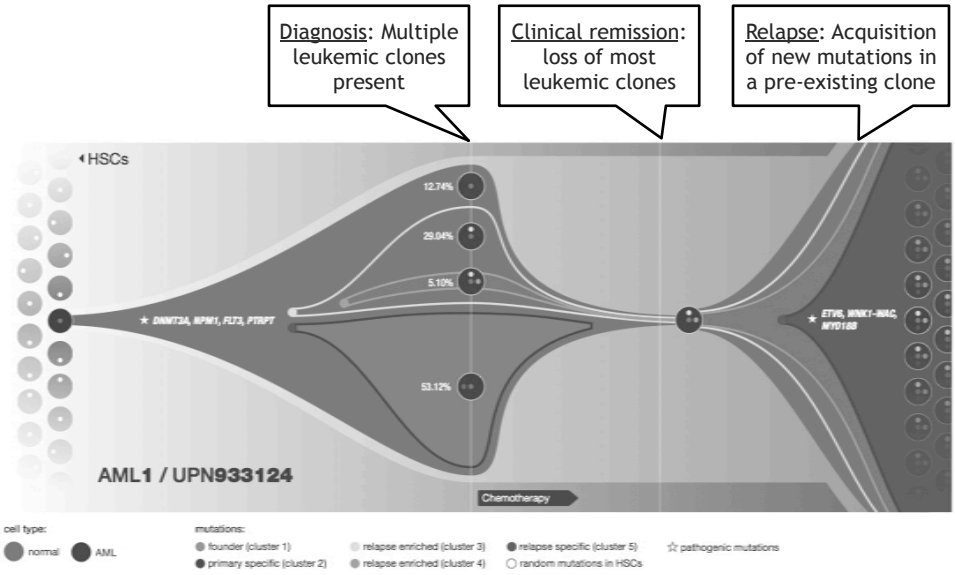
Whole Genome Tumor:Normal Comparison

- Caucasian female, mid-50s at diagnosis
- *De novo* M1 AML
- Family history of AML and lymphoma
- 100% blasts in initial BM sample
- Relapsed and died at 23 months
- Normal cytogenetics
- Informed consent for whole genome sequencing
- Solexa sequencer, 32 bp unpaired reads
- 10 somatic mutations detected



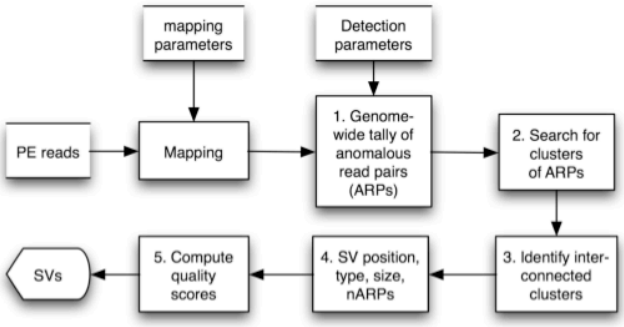
Ley et al., *Nature* 2008

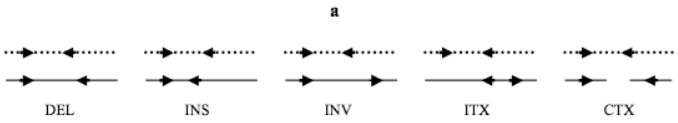
Disease progression model for Patient AML1



BreakDancer: detecting somatic structural variation

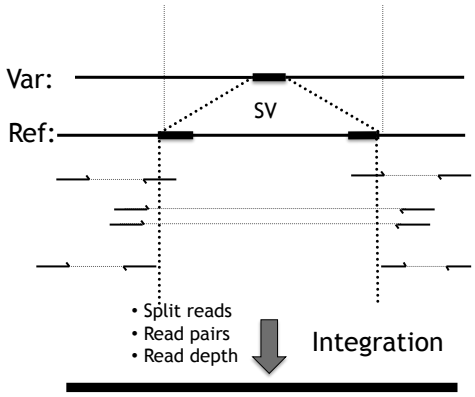
- Read pair analysis with BreakDancer identifies putative SVs based on sets of read pairs that don't map as expected, with respect to distance apart and/or orientation.
- The identified reads are used to produce an assembly, then validated.





K. Chen et al., Nature Methods 6: 677-81 (2009)

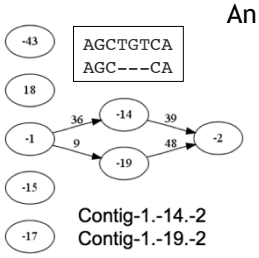
TIGRA_SV: assembling SVs to nucleotide resolution



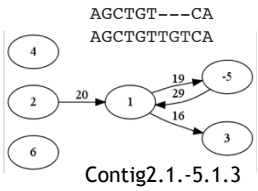
TIGRA_SV targeted local Assembly

Features:
- *de Bruijin* graphic approach
- Multiple Kmer, read threading
- Decode all alleles

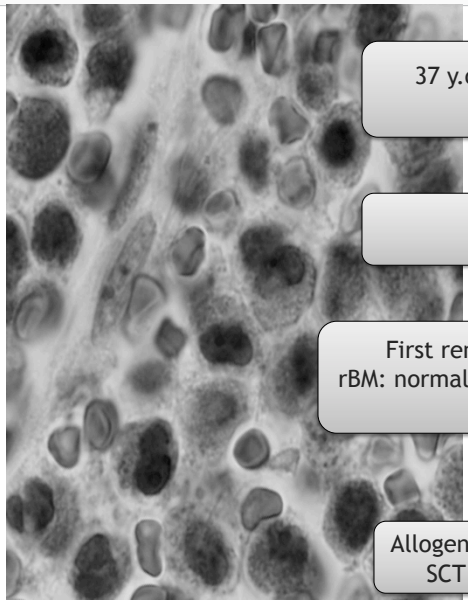
An indel



A tandem duplication



Clinical case: "AML52"



37 y.o. female with *de novo* AML; M3 morphology

↓ Chemo + ATRA

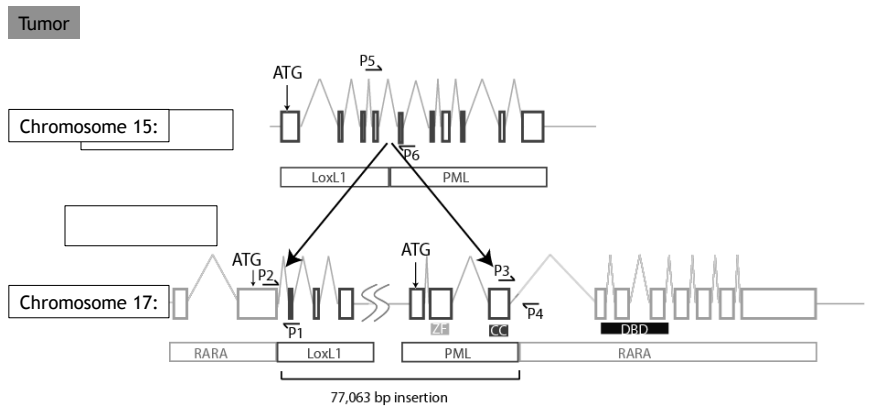
Complex cytogenetics, persistent leukemia

↓ Chemo only

First remission, referred to WU for SCT. rBM: normal morphology, cytogenetics; negative for PML/RARA.

??? → Allogeneic SCT / Consolidation + ATRA

An insertion-derived fusion of PML and RARA



PML-RARA: PML exons 1-3 fused to RARA exons 3-9 (bcr3 variant in frame)

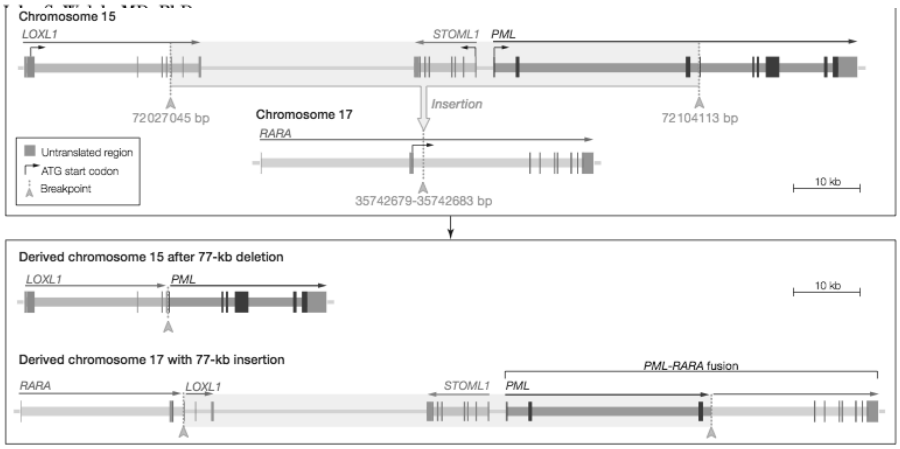
RARA-LoxL1: fusion out of frame

LoxL1-PML: truncated protein - premature stop in novel LoxL1 exon 5a

THE Genome CENTER AT WASHINGTON UNIVERSITY

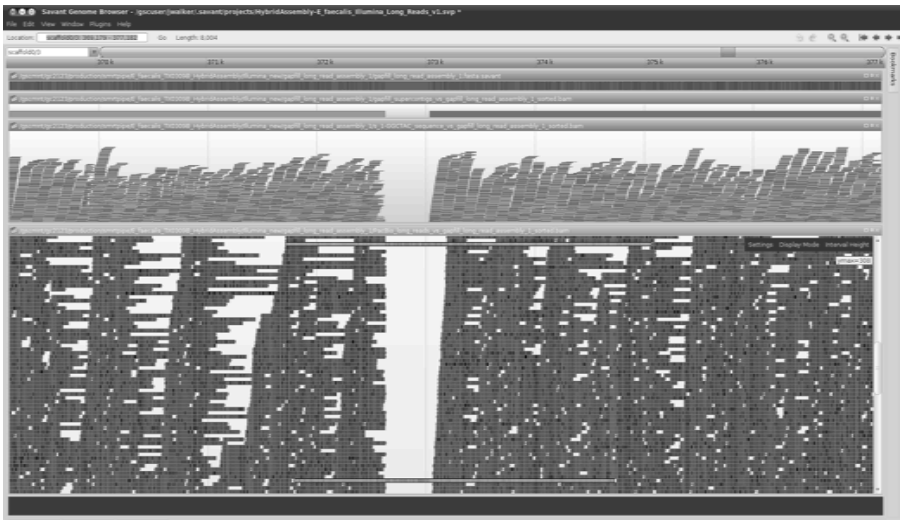
PRELIMINARY COMMUNICATION

Use of Whole-Genome Sequencing to Diagnose a Cryptic Fusion Oncogene



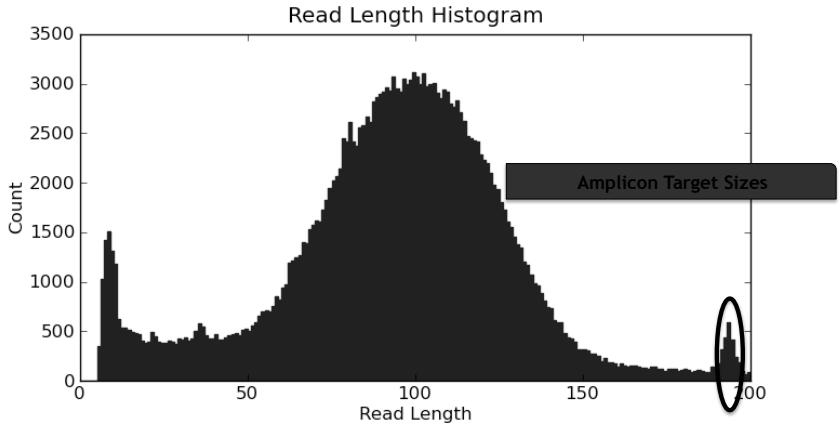
Welch et al., JAMA April 20, 2011

Combining Platforms: *de novo* Assembly



Two VLR PacBio reads contiguate an Illumina assembly gap

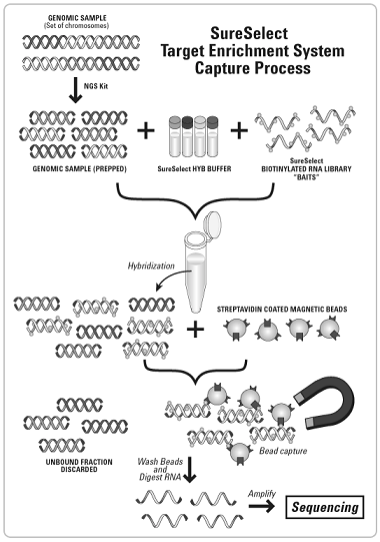
Rapid Genotyping by Ion Torrent



Total Number of Bases [Mbp]	20.35
‣ Number of Q17 Bases [Mbp]	12.49
‣ Number of Q20 Bases [Mbp]	9.66
Total Number of Reads	218,782
Mean Length [bp]	93
Longest Read [bp]	202

Hybrid Capture

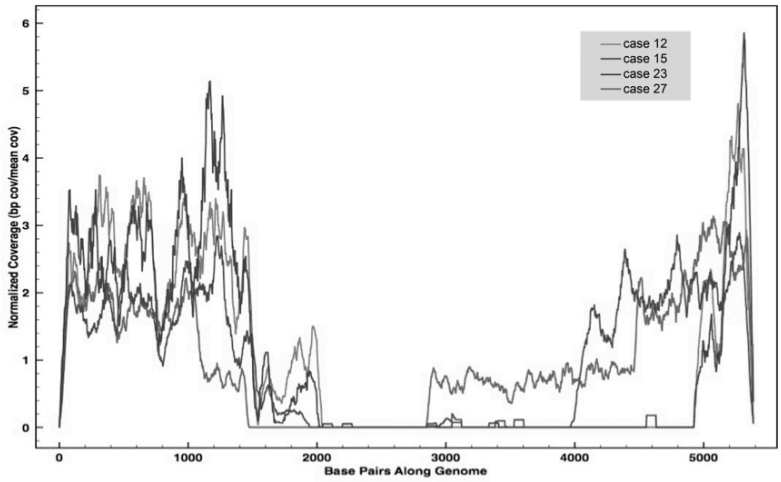
- **Hybrid capture** - fragments from a whole genome library are selected by combining with probes that correspond to most (not all) human exons or gene targets.
- The probe DNAs are biotinylated, making selection from solution with streptavidin magnetic beads an effective means of purification.
- An "**exome**" by definition, is the exons of all genes annotated in the species' reference genome.
- **Custom capture reagents** can be synthesized to target specific loci that may be of interest in a clinical context.



Merkel Cell Polyoma Virus Capture

- Merkel Cell Polyoma virus
  - MCPyV shows frequent genomic deletions and sequence mutations that make it difficult to amplify the virus from cases of MCC by PCR
  - The circular genome does not contain a defined linearization sequence
  - Only FFPE material available for majority of cases
- For proof-of-principle experiments:
  - Biotinylated PCR amplicons designed to target entire 5Kb viral genome
  - Hybrid capture and sequencing
  - Analysis to identify insertion points in human genomes

Viral Coverage Plots (4 FFPE Samples)



Duncavage et al., JMD 2011

Finding the Junction (Integration Site) using SLOPE

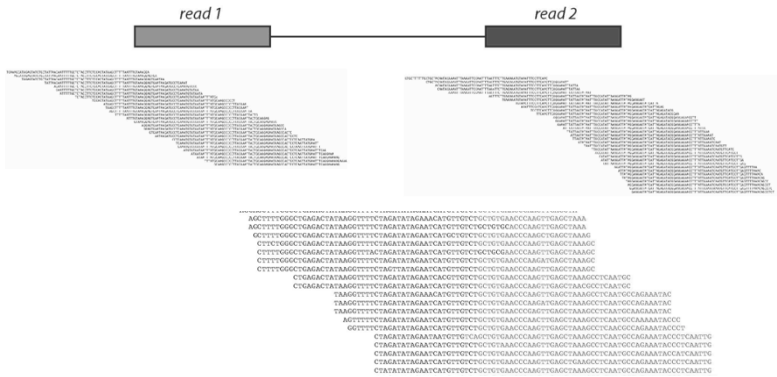


Table 3. MCC Case Clinical Characteristics, PCR-Verified Viral Insertion Sites, and Viral Deletions

Sample	Type	Age of block (years)	Sex	Site	3′ insertion site	5′ insertion site	Viral genomic deletion size
12	Primary	6	M	Buttocks	ch8: 65568962	ch8: 65566806	3.0 kb
23	Metastasis	5	M	Back	ch8: 65568962	ch8: 65566806	3.0 kb
27	Metastasis	2	F	Right arm	ch9: 121417276	undetermined	2.4 kb
15	Metastasis	6	M	Small bowel	ch6: 19684666	ch6: 19684859	1.3 kb

RNA Sequencing

RNA isolate

polyA priming, RT, ds DNA

Fragment, RT w/randoms

SAGE tags

Size selection for nc RNA classes

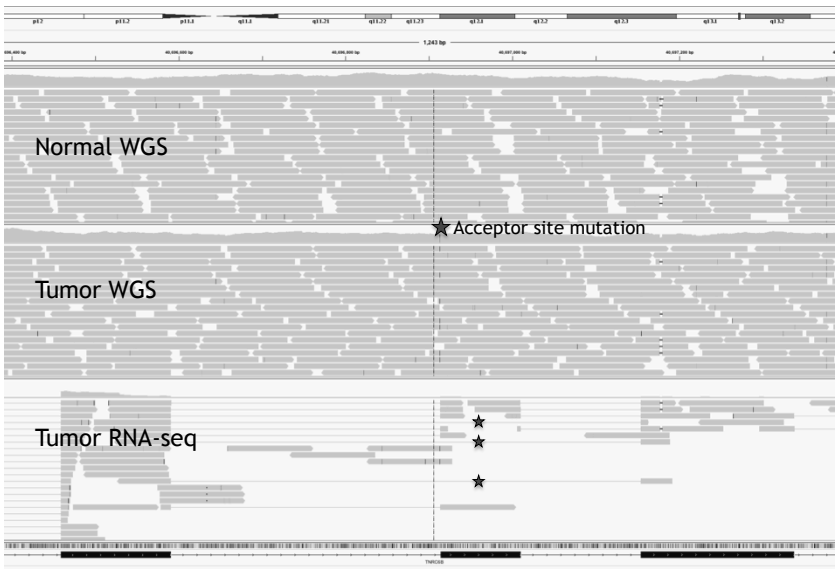
Adapter-ligated fragments for next-gen sequencing

↓

Alignment to reference database & discovery

-Expression levels
-Novel splice isoforms
-Allelic bias in transcription
-Fusion transcripts

TNRC6B splice site mutation -> alt. splicing



Metastatic breast cancer (to brain)

Human Microbiome Project

Goals:
-Defining Core Microbiomes in "healthy" subjects
-Catalog changes in Microbiomes with disease

Subjects → Samples → Microbial Communities

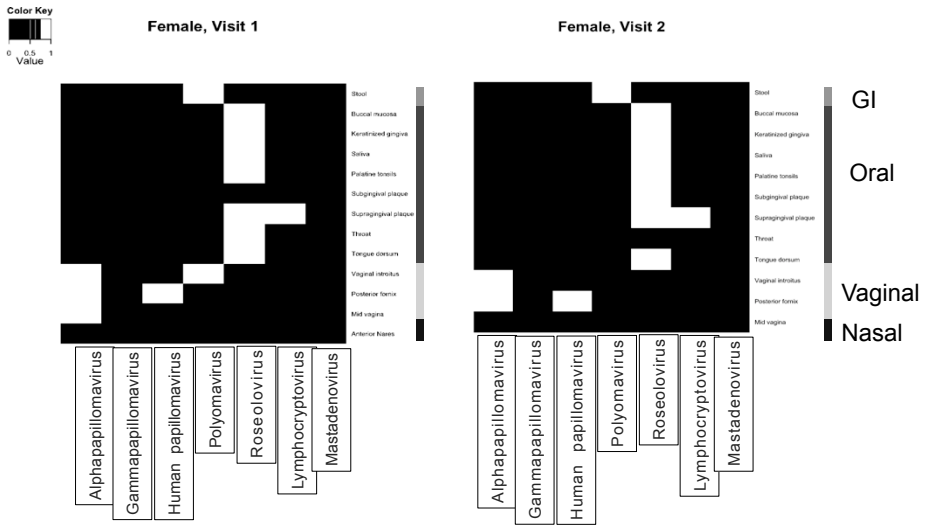
Microbial Communities → Community Phenotypes

Microbial Communities → Sources of strains → Reference Sequences → Microbial Communities

Microbial Communities → Metagenomics → WGS, 16S rRNA

Data Coordination, Analysis and Concordance to Clinical Data

Stability of virome over time



Kristine Wylie, WUGI

400 MRSA Genomes

Conclusions

- 2nd and 3rd generation sequencing instruments are revolutionizing biological research.
- Earliest impacts have been on cancer genomics and metagenomics.
- The extreme need for bioinformatics-based analytical approaches to interpret these large data sets has revitalized the field and introduced statistical and mathematical rigor.
- Integration across data sets from DNA, RNA, methylation, proteomics, etc. presents the next challenge but provides comprehensive analytical power to inform biology.
- With newer instruments, clinical applications have potential for implementation, with appropriate interpretive algorithms.

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