The Resurgence of Reference Quality Genomes using 3rd Gen Sequencing

Michael Schatz

Dec 9, 2014

American Museum of Natural History

Schatzlab Overview

Informatics

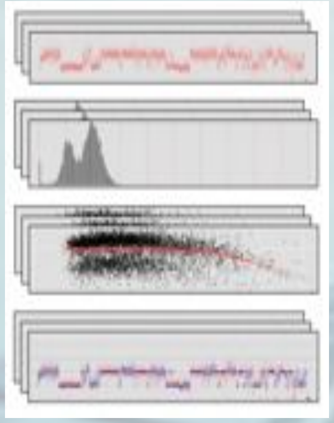
Ultra-large scale
biocomputing

Blood *et al.* (2014)
Schatz *et al.* (2013)

Human Genetics

Autism, Cancer,
Psychiatric Disorders

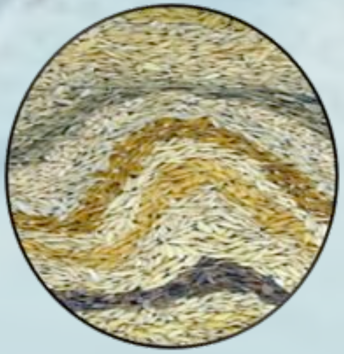
Narzisi *et al.* (2014)
Iossifov *et al.* (2014)



Biotechnology

Single Cell &
Single Molecule
Analysis

Garvin *et al.* (2014)
Roberts *et al.* (2013)



Plant Biology

Genomes &
Transcriptomes

Schatz *et al.* (2014)
Ming *et al.* (2013)

Outline

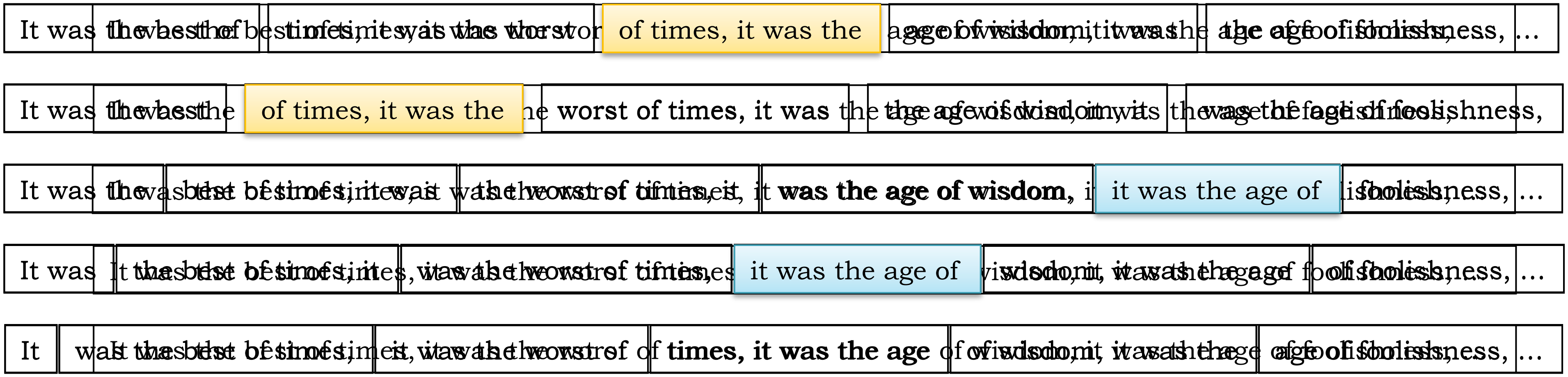
1. Assembly theory
    1. Assembly by analogy
    2. De Bruijn and Overlap graph
    3. Coverage, read length, errors, and repeats

2. Sequencing and Assembly options
    1. Illumina/ALLPATHS-LG
    2. Pacific Biosciences
    3. Oxford Nanopore

3. Summary & Recommendations

Shredded Book Reconstruction

- Dickens accidentally shreds the first printing of A Tale of Two Cities
  - Text printed on 5 long spools



- How can he reconstruct the text?
  - 5 copies x 138, 656 words / 5 words per fragment = 138k fragments
  - The short fragments from every copy are mixed together
  - Some fragments are identical

Greedy Reconstruction

It was the best of

age of wisdom, it was

best of times, it was

it was the age of

it was the age of

it was the worst of

of times, it was the

of times, it was the

of wisdom, it was the

the age of wisdom, it

the best of times, it

the worst of times, it

times, it was the age

times, it was the worst

was the age of wisdom,

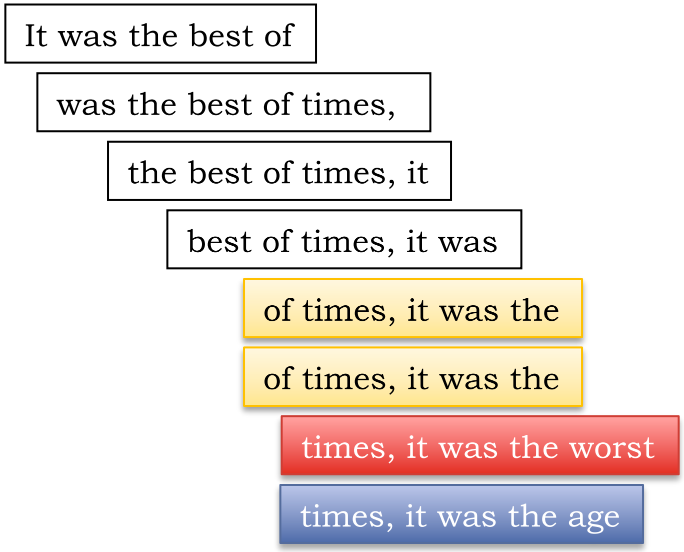
was the age of foolishness,

was the best of times,

was the worst of times,

wisdom, it was the age

worst of times, it was



It was the best of

was the best of times,

the best of times, it

best of times, it was

of times, it was the

of times, it was the

times, it was the worst

times, it was the age

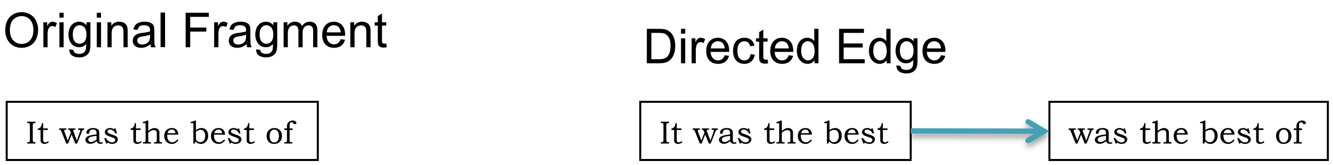
The repeated sequence make the correct reconstruction ambiguous

- It was the best of times, it was the [worst/age]

Model the assembly problem as a graph problem

de Bruijn Graph Construction

- $D_k = (V,E)$
  - V = All length-k subfragments $(k < l)$
  - E = Directed edges between consecutive subfragments
    - Nodes overlap by k-1 words



Original Fragment

It was the best of

Directed Edge

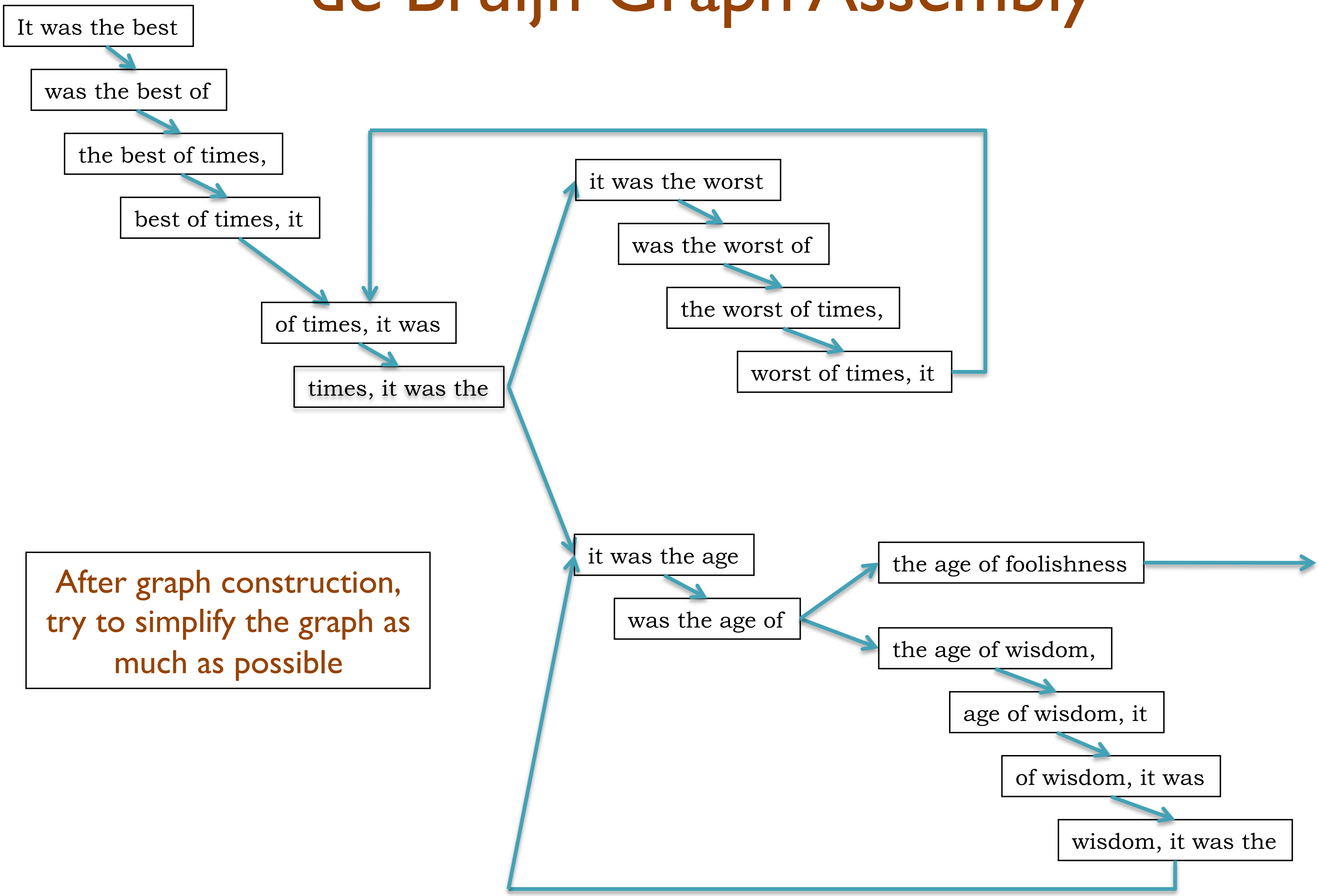
It was the best

was the best of

- Locally constructed graph reveals the global sequence structure
  - Overlaps between sequences implicitly computed

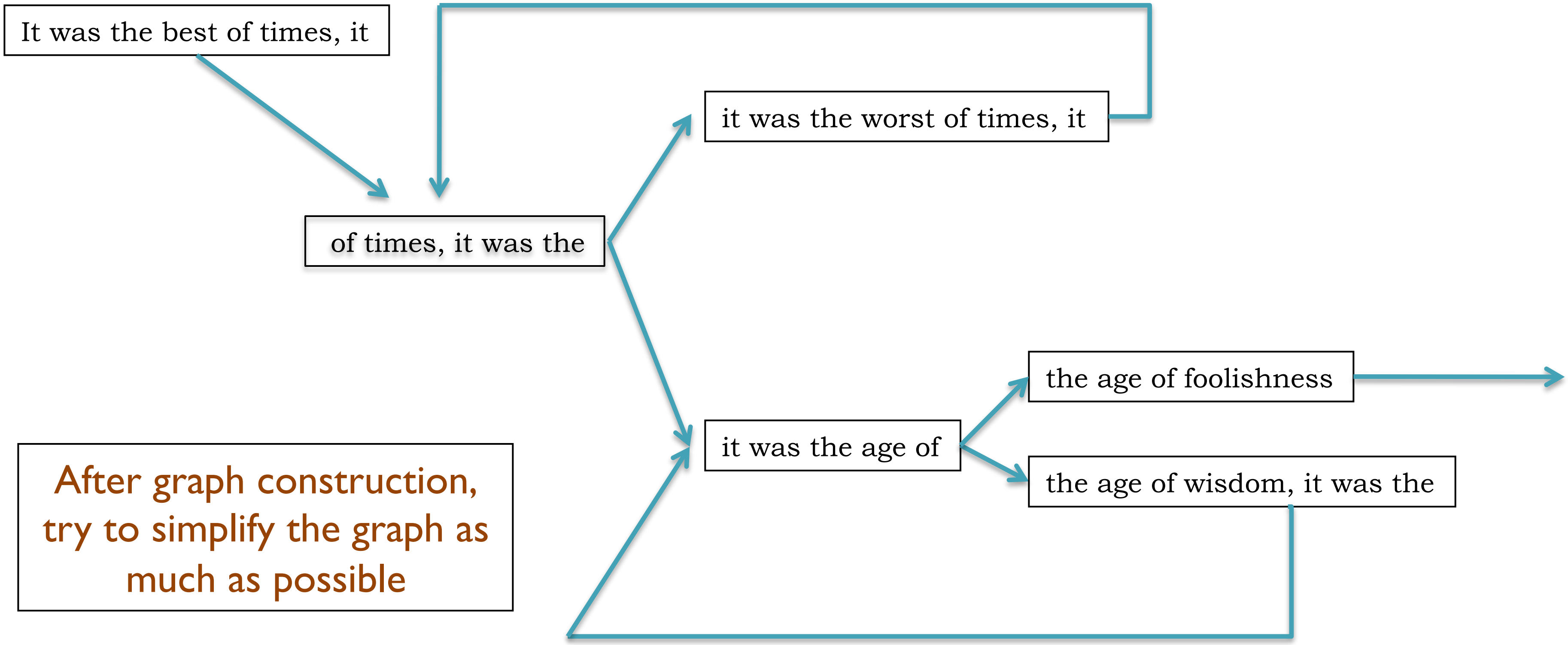
de Bruijn, 1946
Idury and Waterman, 1995
Pevzner, Tang, Waterman, 2001

de Bruijn Graph Assembly



After graph construction, try to simplify the graph as much as possible

de Bruijn Graph Assembly



It was the best of times, it

of times, it was the

it was the worst of times, it

it was the age of

the age of foolishness

the age of wisdom, it was the

After graph construction, try to simplify the graph as much as possible

The full tale

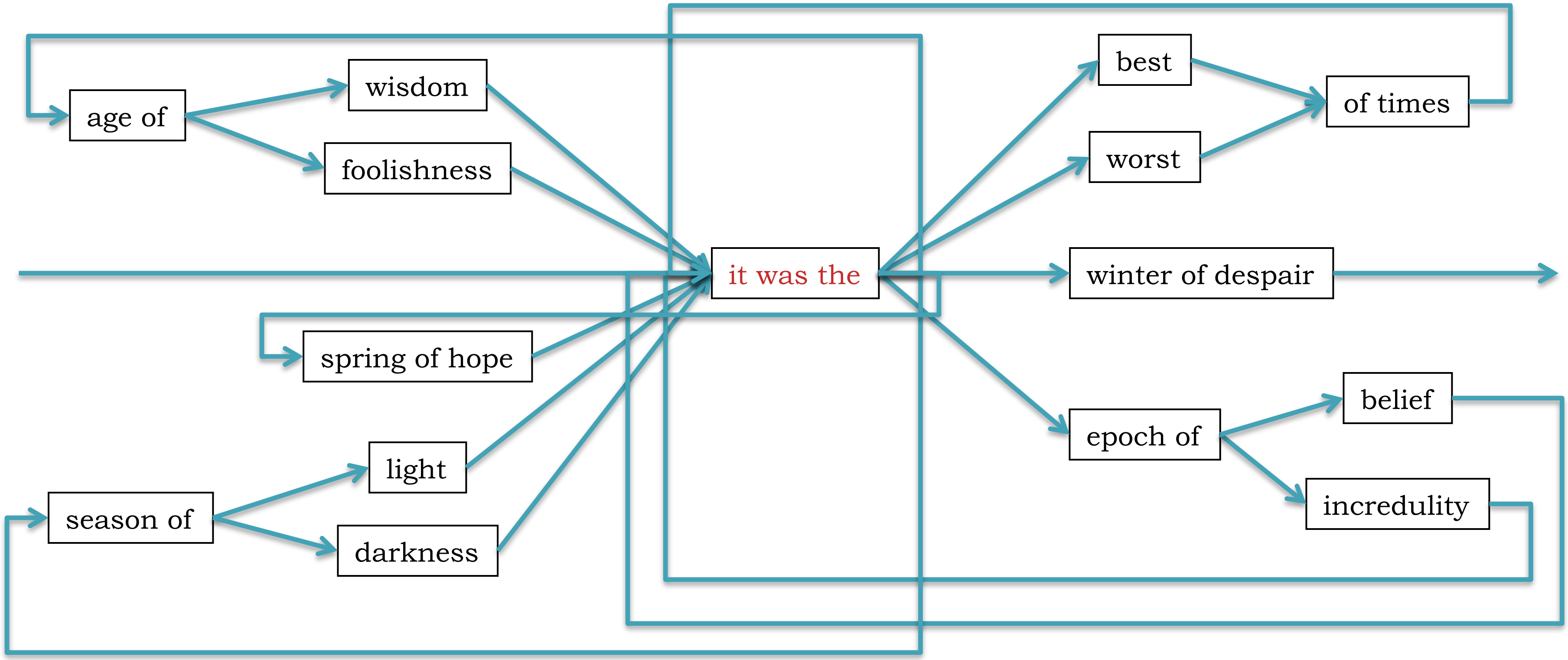
… it was the best of times it was the worst of times …

… it was the age of wisdom it was the age of foolishness …

… it was the epoch of belief it was the epoch of incredulity …

… it was the season of light it was the season of darkness …

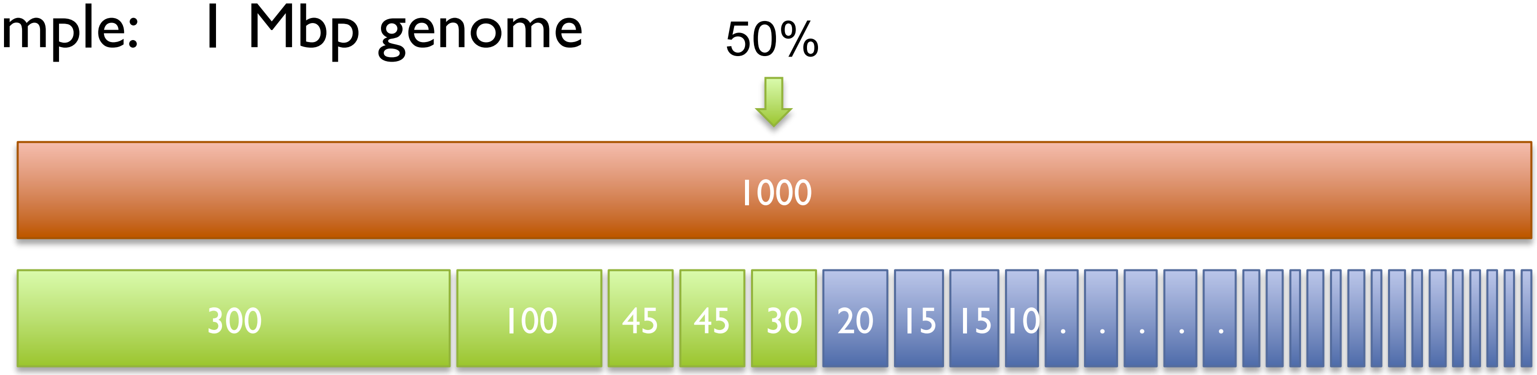
… it was the spring of hope it was the winder of despair …



N50 size

Def: 50% of the genome is in contigs as large as the N50 value

Example: 1 Mbp genome



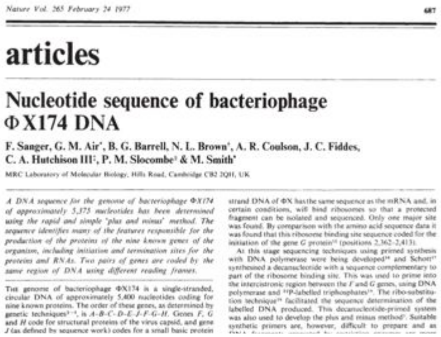
N50 size = 30 kbp

(300k+100k+45k+45k+30k = 520k >= 500kbp)

A greater N50 is indicative of improvement in every dimension:

- Better resolution of genes and flanking regulatory regions
- Better resolution of transposons and other complex sequences
- Better resolution of chromosome organization
- Better sequence for all downstream analysis

Milestones in Genome Assembly



Nature Vol. 265 February 24 1977 687

articles

Nucleotide sequence of bacteriophage ΦX174 DNA

F. Sanger, G. M. Air*, B. G. Barrell, N. L. Brown†, A. R. Coulson, J. C. Fiddes, C. A. Hutchison III‡, P. M. Slocombe† & M. Smith*

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

A DNA sequence for the genome of bacteriophage ΦX174 of approximately 5,375 nucleotides has been determined using the rapid and simple 'plus and minus' method. The sequence identifies many of the features responsible for the production of the proteins of the nine known genes of the organism, including initiation and termination sites for the proteins and RNAs. Two pairs of genes are coded by the same region of DNA using different reading frames.

THE genome of bacteriophage ΦX174 is a single-stranded, circular DNA of approximately 5,400 nucleotides coding for nine known proteins. The order of these genes, as determined by genetic techniques[1-4], is *A-B-C-D-E-J-F-G-H*. Genes *F*, *G* and *H* code for structural proteins of the virus capsid, and gene *J* (as defined by sequence work) codes for a small basic protein

strand DNA of ΦX has the same sequence as the mRNA and, in certain conditions, will bind ribosomes so that a protected fragment can be isolated and sequenced. Only one major site was found. By comparison with the amino acid sequence data it was found that this ribosome binding site sequence coded for the initiation of the gene *G* protein[11] (positions 2,362-2,413).

At this stage sequencing techniques using primed synthesis with DNA polymerase were being developed[14] and Schott[15] synthesised a decanucleotide with a sequence complementary to part of the ribosome binding site. This was used to prime into the intercistronic region between the *F* and *G* genes, using DNA polymerase and ^{32}P-labelled triphosphates[16]. The ribo-substitution technique[16] facilitated the sequence determination of the labelled DNA produced. This decanucleotide-primed system was also used to develop the plus and minus method[2]. Suitable synthetic primers are, however, difficult to prepare and as

1977. Sanger *et al.*
1st Complete Organism
5375 bp

1995. Fleischmann *et al.*
1st Free Living Organism
TIGR Assembler. 1.8Mbp

1998. C.elegans SC
1st Multicellular Organism
BAC-by-BAC Phrap. 97Mbp

2000. Myers *et al.*
1st Large WGS Assembly.
Celera Assembler. 116 Mbp

2001.Venter *et al.*, IHGSC
Human Genome
Celera Assembler/GigaAssembler. 2.9 Gbp

2010. Li *et al.*
1st Large SGS Assembly.
SOAPdenovo 2.2 Gbp

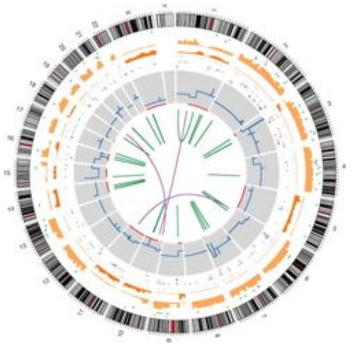
Like Dickens, we must computationally reconstruct a genome from short fragments

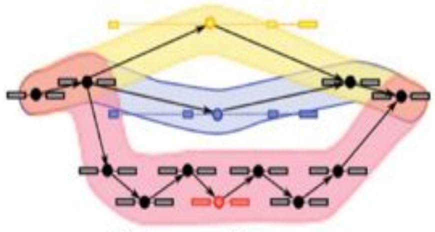
Assembly Applications

- Novel genomes

- Metagenomes

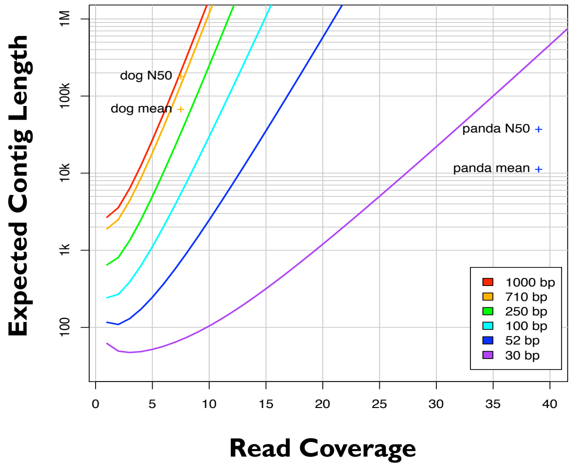
- Sequencing assays
  - Structural variations
  - Transcript assembly
  - …





Ingredients for a good assembly

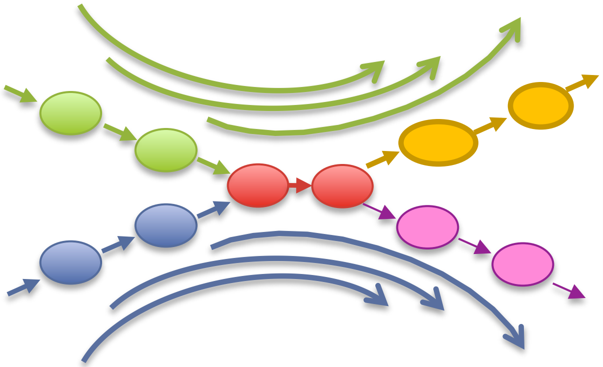
Coverage



High coverage is required

- Oversample the genome to ensure every base is sequenced with long overlaps between reads
- Biased coverage will also fragment assembly

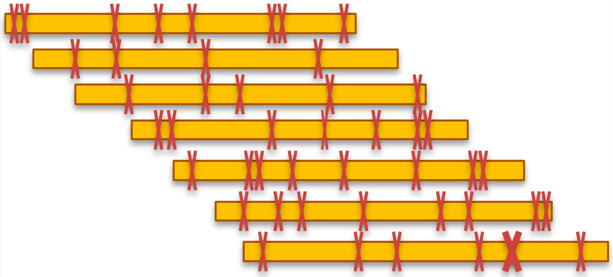
Read Length



Reads & mates must be longer than the repeats

- Short reads will have ***false overlaps*** forming hairball assembly graphs
- With long enough reads, assemble entire chromosomes into contigs

Quality

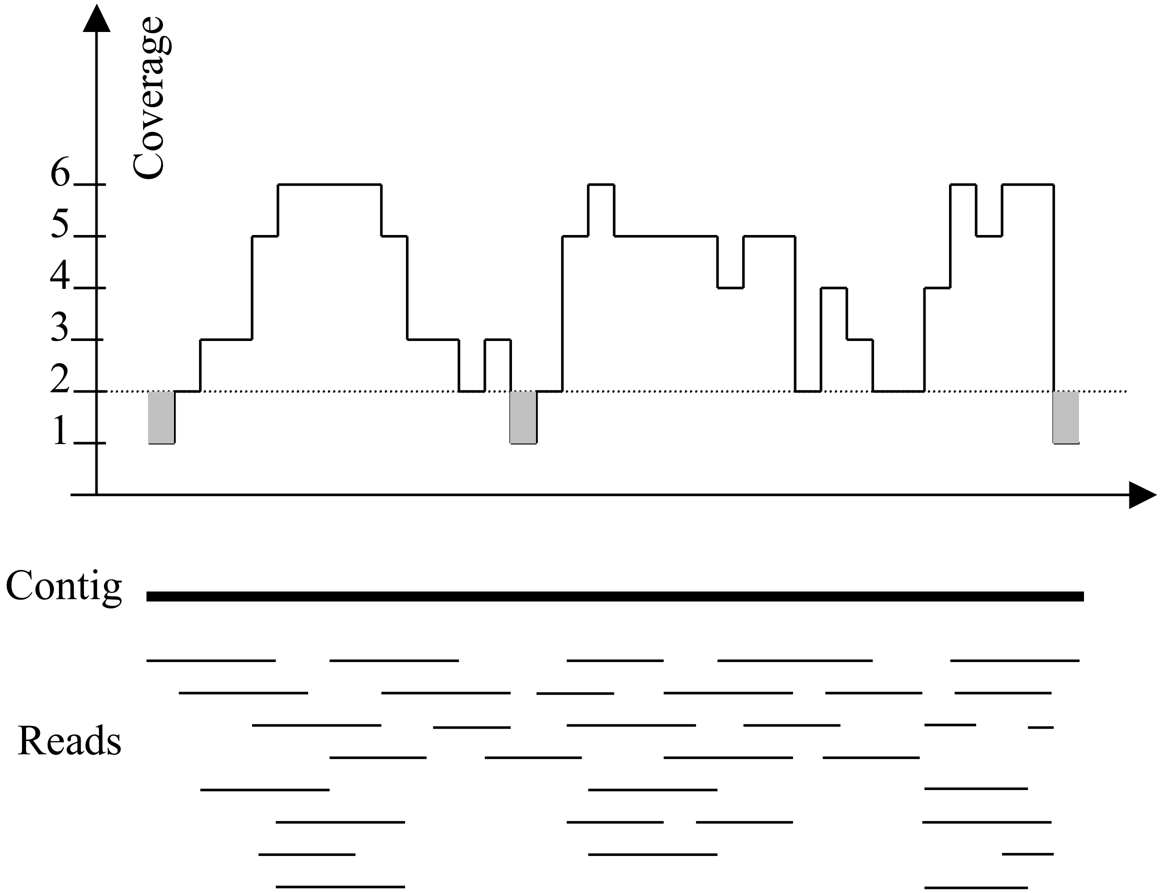


Errors obscure overlaps

- Reads are assembled by finding kmers shared in pair of reads
- High error rate requires very short seeds, increasing complexity and forming assembly hairballs

Current challenges in *de novo* plant genome sequencing and assembly
Schatz MC, Witkowski, McCombie, WR (2012) *Genome Biology*. 12:243

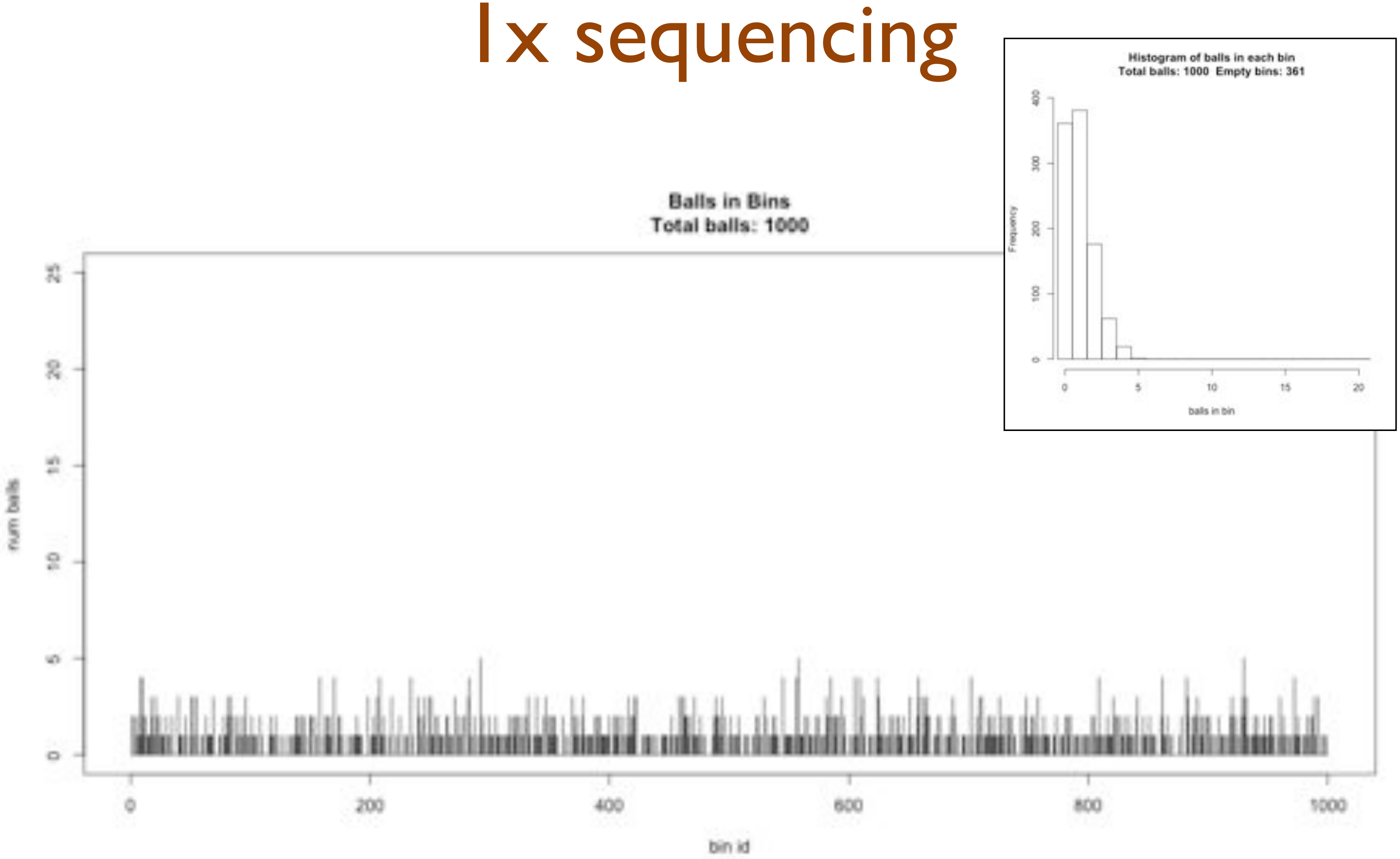
Typical sequencing coverage



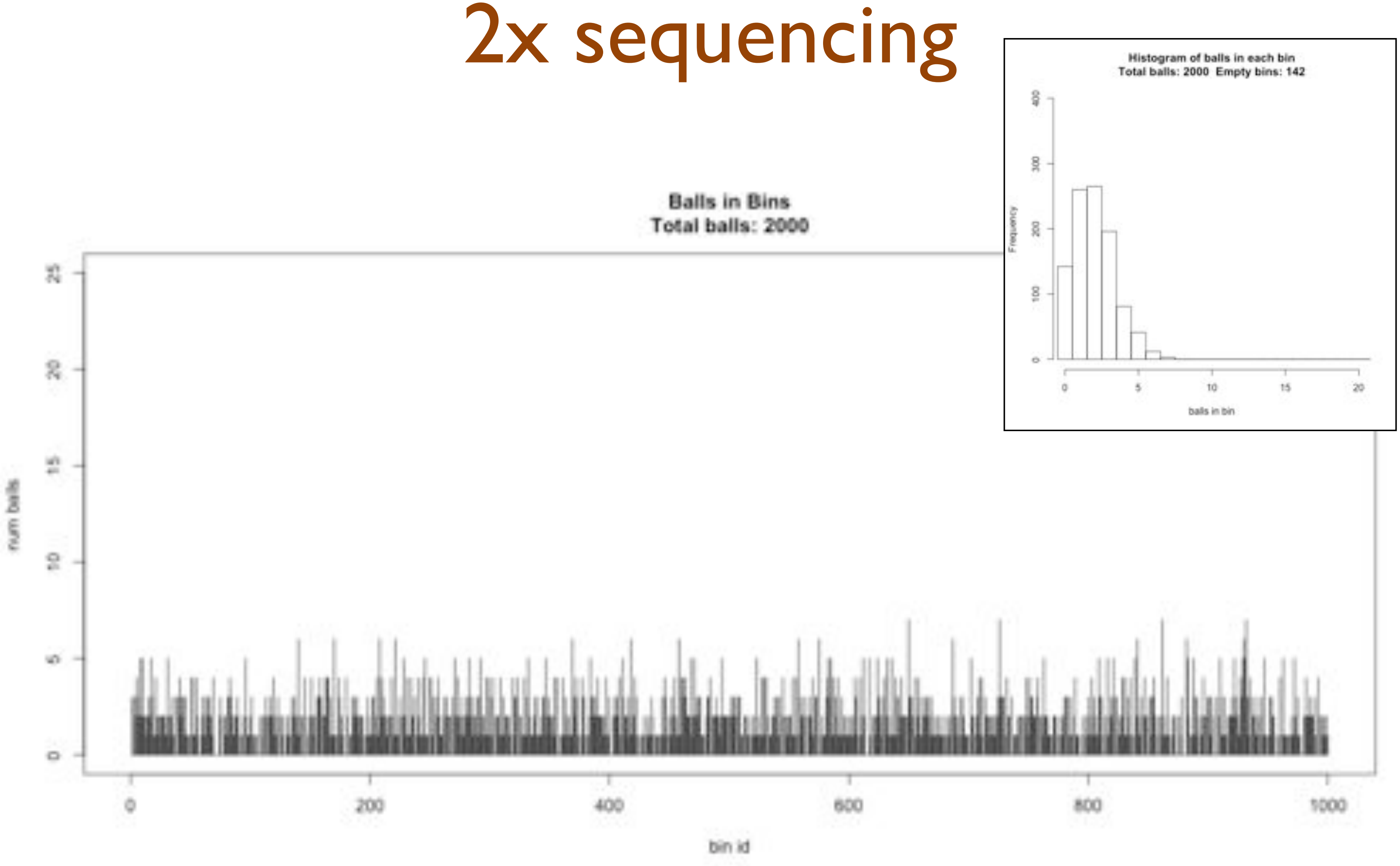
Imagine raindrops on a sidewalk

We want to cover the entire sidewalk but each drop costs $1

1x sequencing

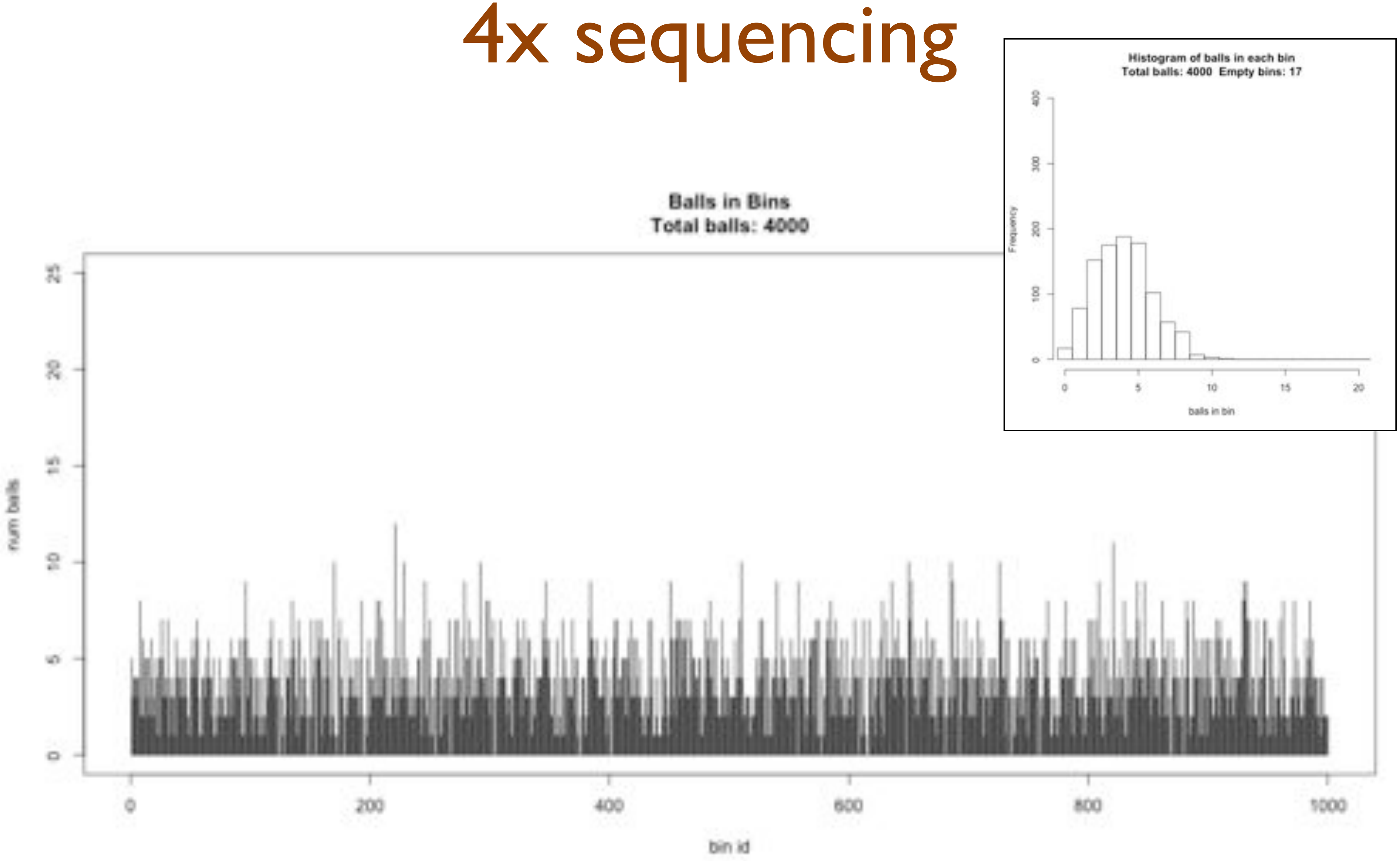


2x sequencing

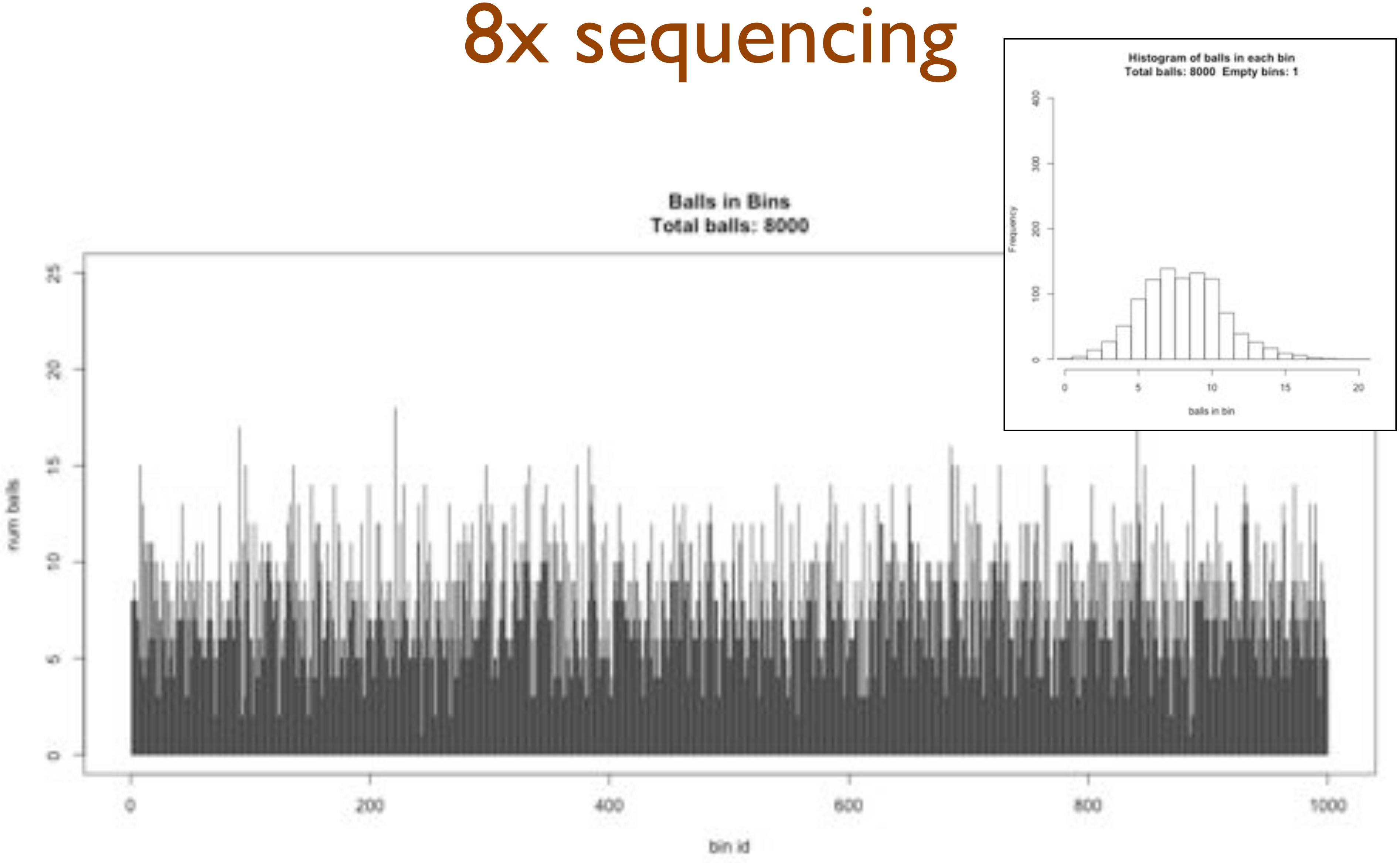

Balls in Bins
Total balls: 2000

Histogram of balls in each bin
Total balls: 2000 Empty bins: 142

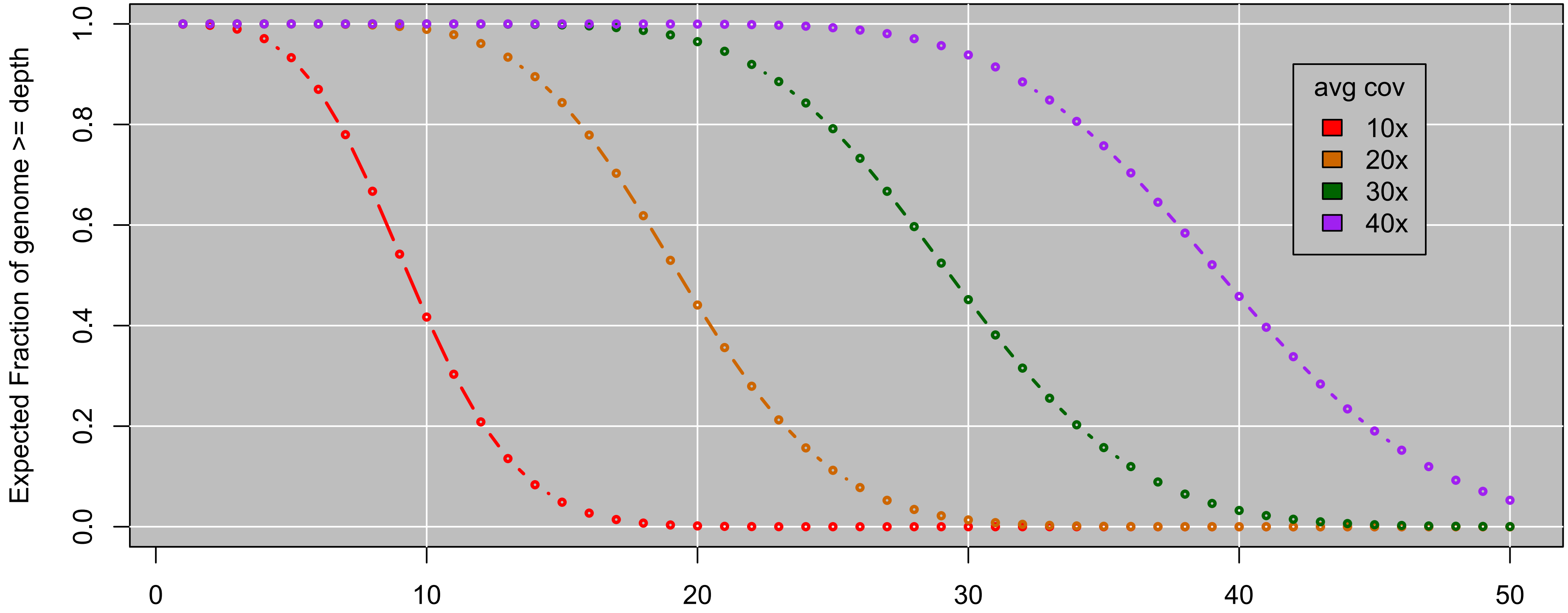
4x sequencing



8x sequencing



Genome Coverage Distribution

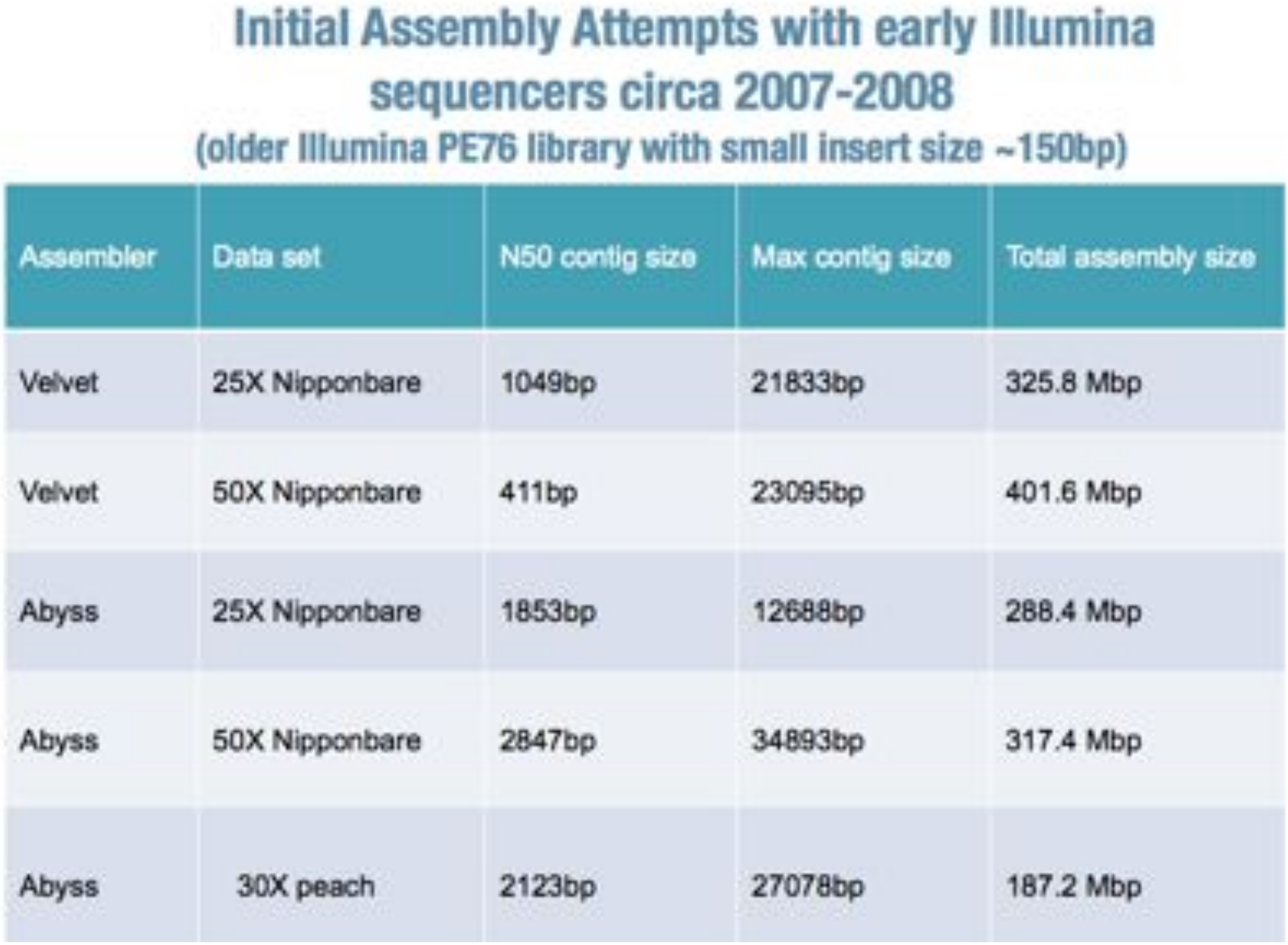


Expect Poisson distribution on depth

- Standard Deviation = sqrt(cov)

This is the mathematically model => reality may be much worse

- Double your coverage for diploid genomes
- Can use somewhat lower coverage in a population to find common variants



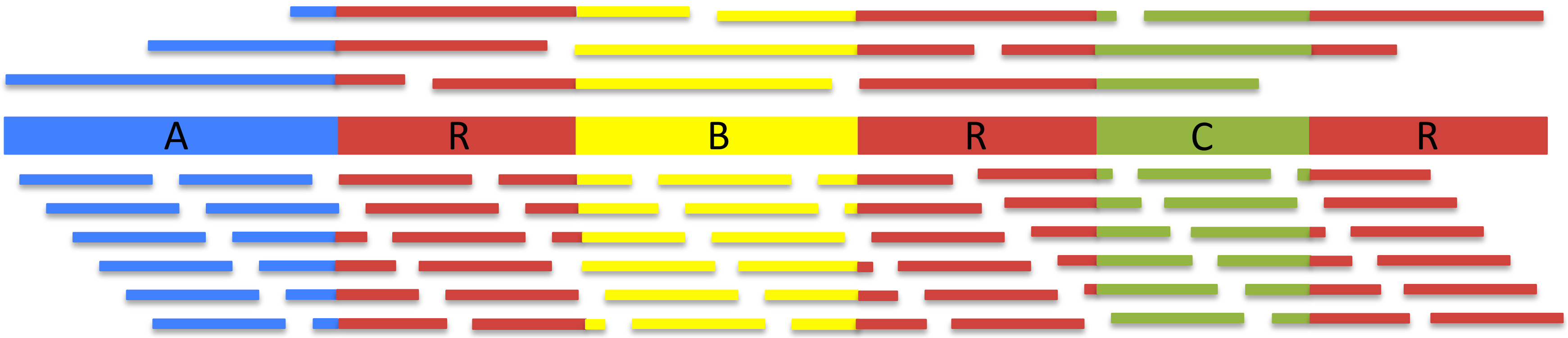
Initial Assembly Attempts with early Illumina sequencers circa 2007-2008

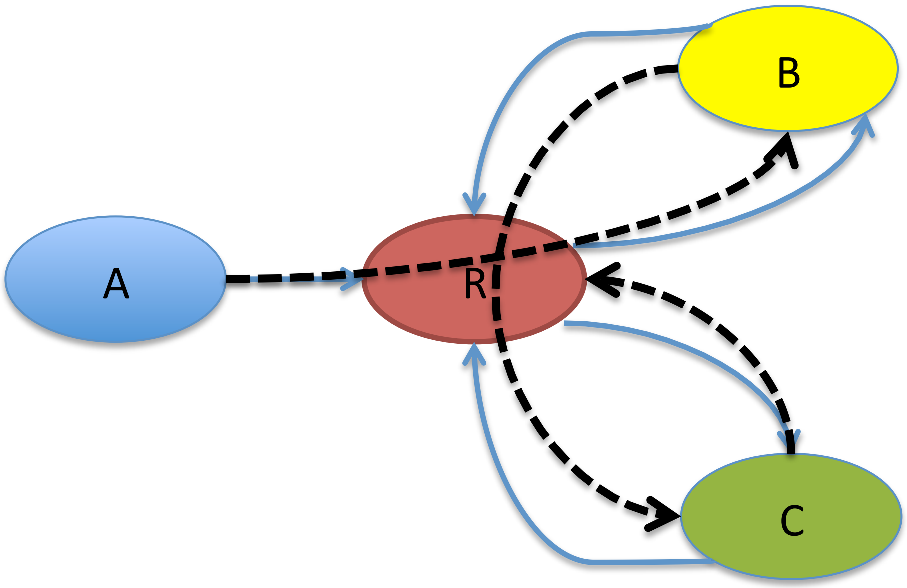
(older Illumina PE76 library with small insert size ~150bp)

Assembler	Data set	N50 contig size	Max contig size	Total assembly size
Velvet	25X Nipponbare	1049bp	21833bp	325.8 Mbp
Velvet	50X Nipponbare	411bp	23095bp	401.6 Mbp
Abyss	25X Nipponbare	1853bp	12688bp	288.4 Mbp
Abyss	50X Nipponbare	2847bp	34893bp	317.4 Mbp
Abyss	30X peach	2123bp	27078bp	187.2 Mbp

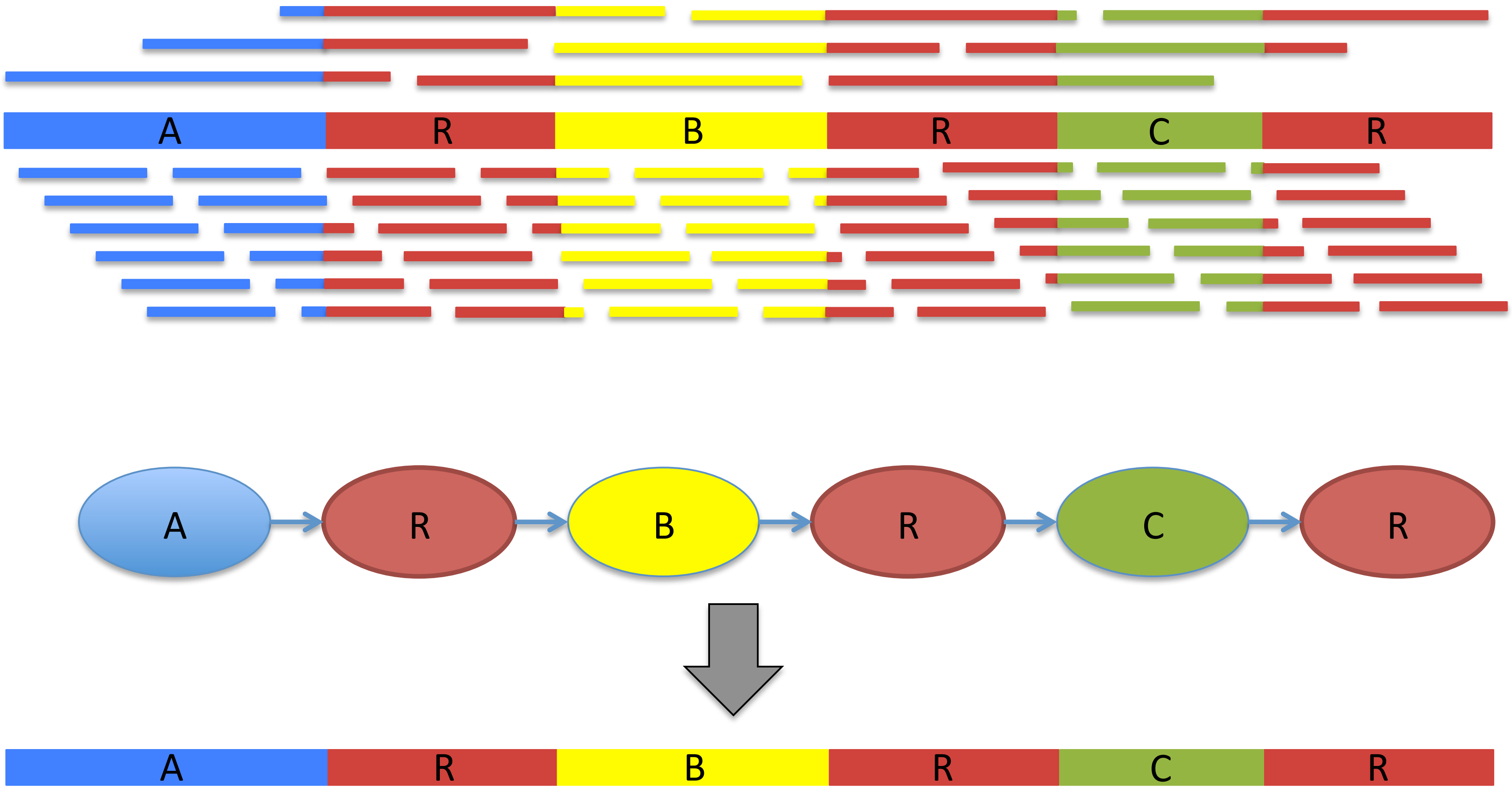
W.R. McCombie

Assembly Complexity



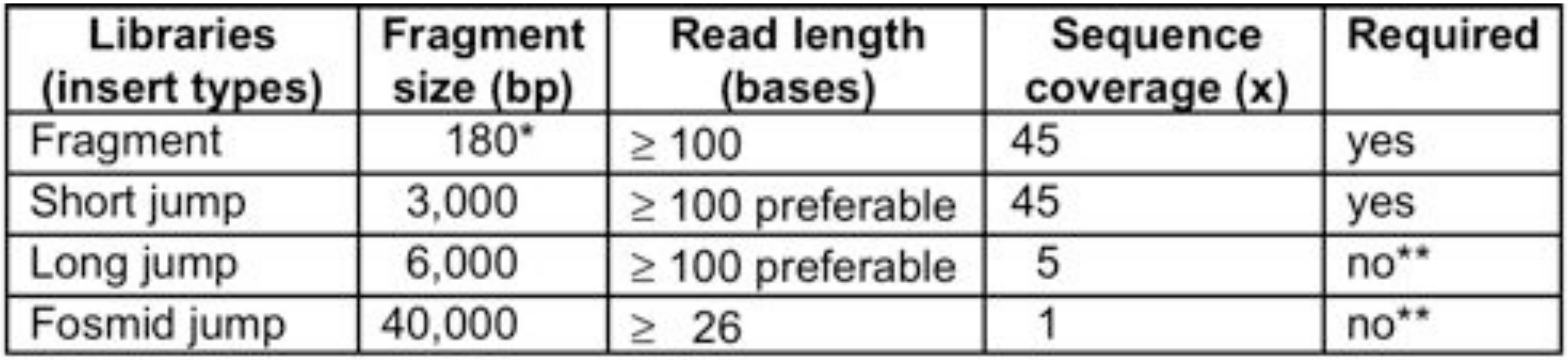


Assembly Complexity

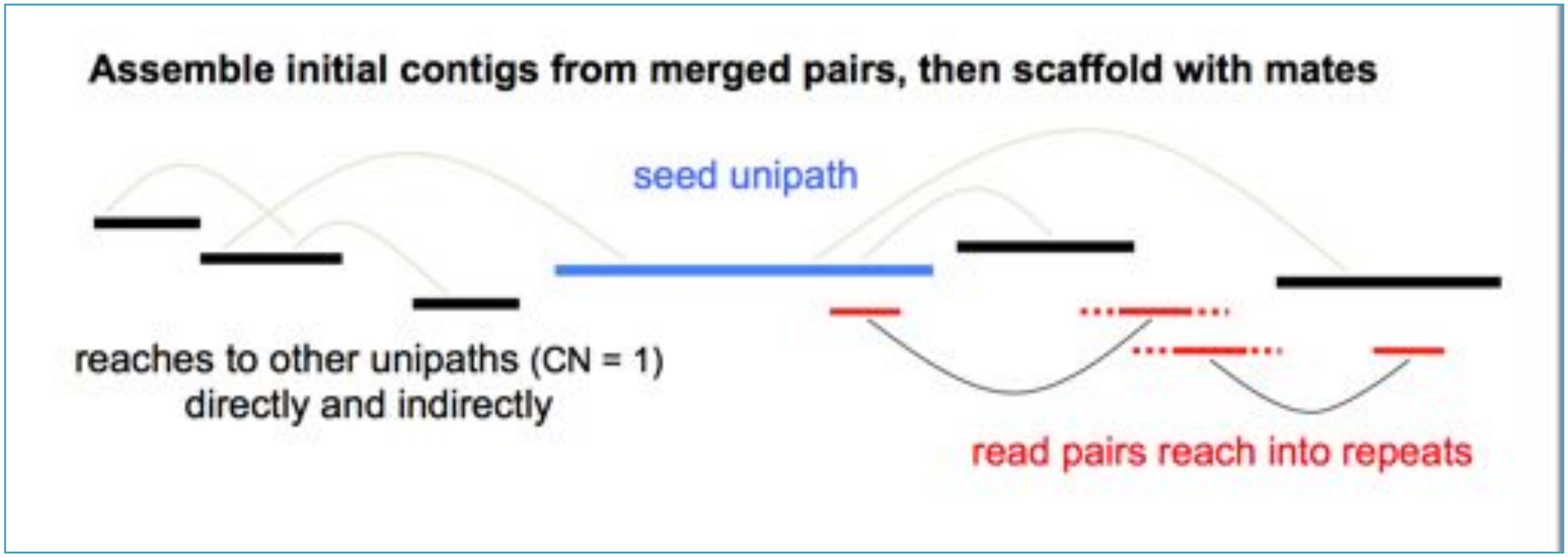


The advantages of SMRT sequencing
Roberts, RJ, Carneiro, MO, Schatz, MC (2013) *Genome Biology.* 14:405

Short Read Assembly with ALLPATHS



Libraries (insert types)	Fragment size (bp)	Read length (bases)	Sequence coverage (x)	Required
Fragment	180*	≥ 100	45	yes
Short jump	3,000	≥ 100 preferable	45	yes
Long jump	6,000	≥ 100 preferable	5	no**
Fosmid jump	40,000	≥ 26	1	no**



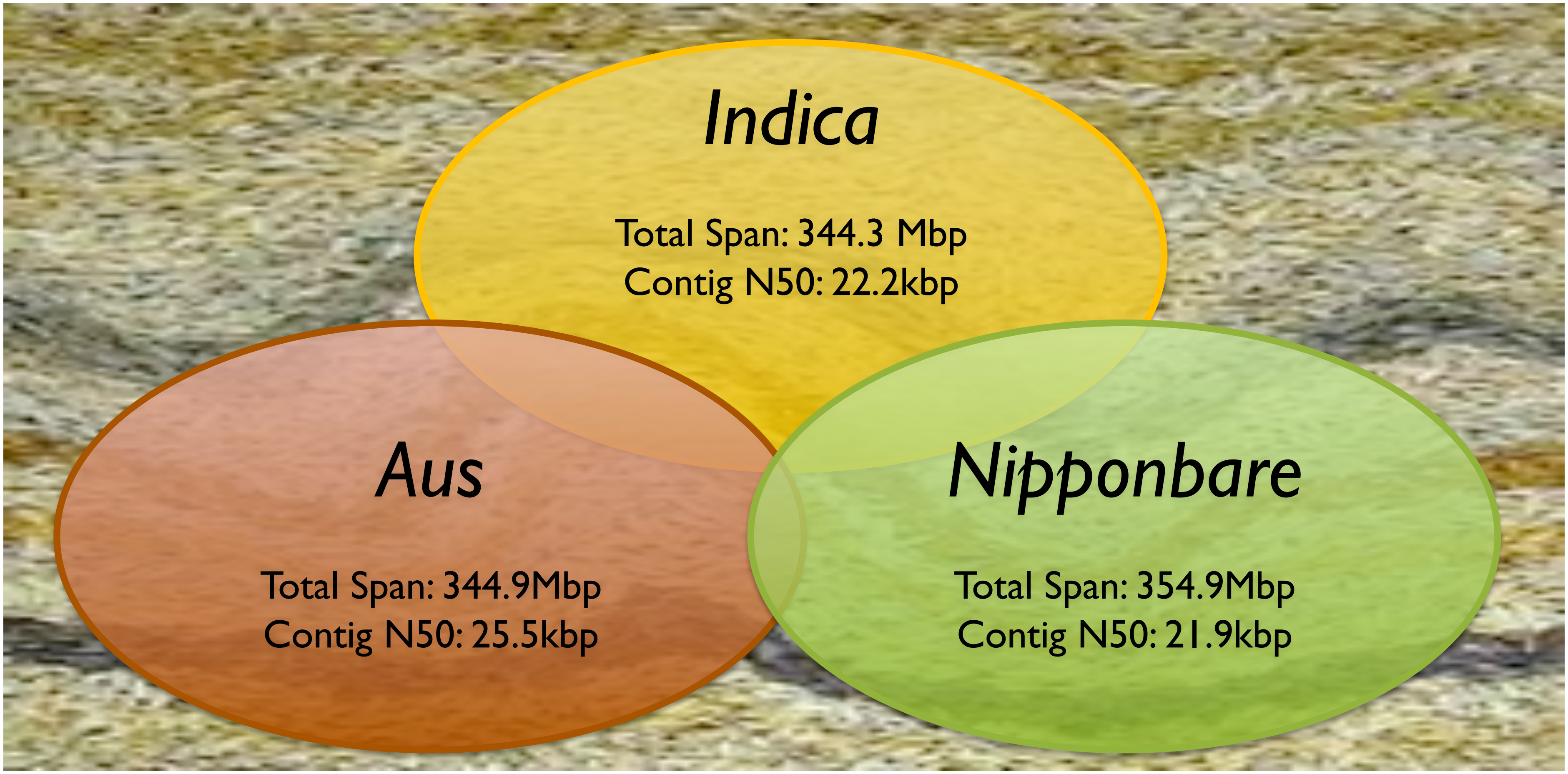
Assemble initial contigs from merged pairs, then scaffold with mates

seed unipath
reaches to other unipaths (CN = 1)
directly and indirectly
read pairs reach into repeats

High-quality draft assemblies of mammalian genomes from massively parallel sequence data

Gnerre et al (2010) *PNAS*. doi: 10.1073/pnas.1017351108

Population structure of *Oryza sativa*



Indica

Total Span: 344.3 Mbp
Contig N50: 22.2kbp

Aus

Total Span: 344.9Mbp
Contig N50: 25.5kbp

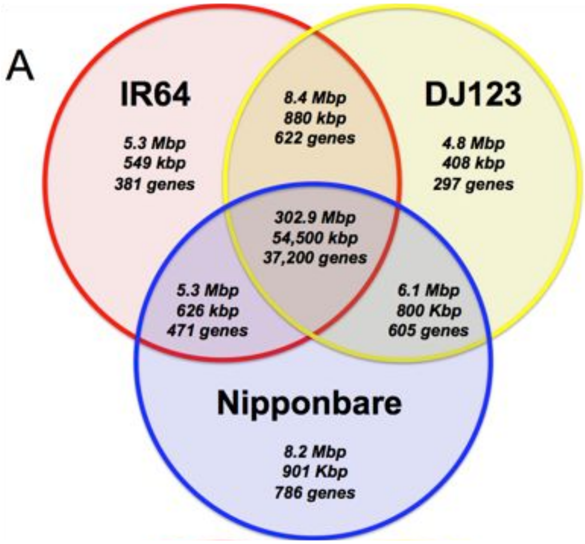
Nipponbare

Total Span: 354.9Mbp
Contig N50: 21.9kbp

Whole genome de novo assemblies of three divergent strains of rice (*O. sativa*) documents novel gene space of *aus* and *indica*
Schatz, Maron, Stein et a*l* (2014) *Genome Biology.* 15:506 doi:10.1186/s13059-014-0506-z

Oryza sativa Gene Diversity

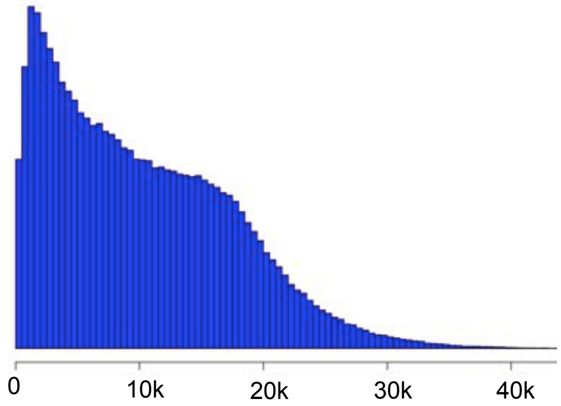
- Very high quality representation of the "gene-space"
  - Overall identity ~99.9%
  - Less than 1% of exonic bases missing
- Genome-specific genes enriched for disease resistance
  - Reflects their geographic and environmental diversity
- Assemblies fragmented at (high copy) repeats
  - Difficult to identify full length gene models and regulatory features



Overall sequence content
In each sector, the top number is the total number of base pairs, the middle number is the number of exonic bases, and the bottom is the gene count. If a gene is partially shared, it is assigned to the sector with the most exonic bases.

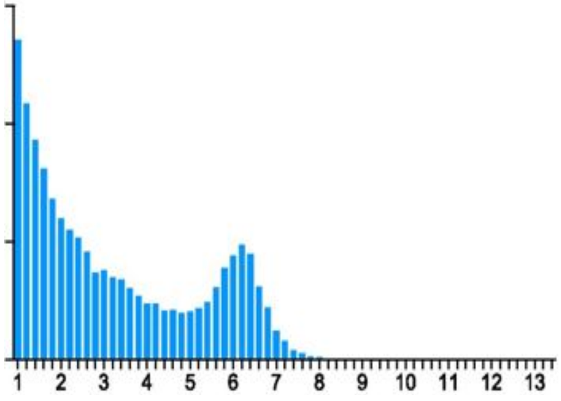
Long Read Sequencing Technology

PacBio RS II



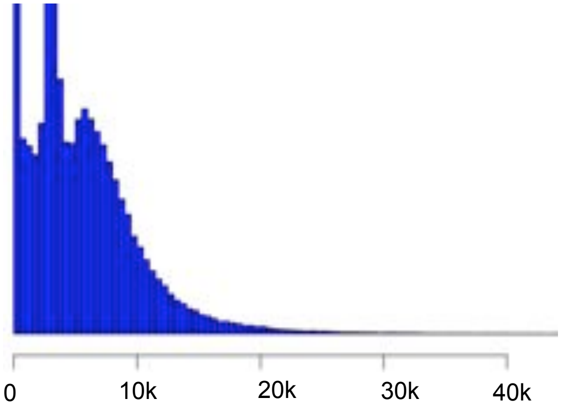
CSHL/PacBio

Moleculo



(Voskoboynik et al. 2013)

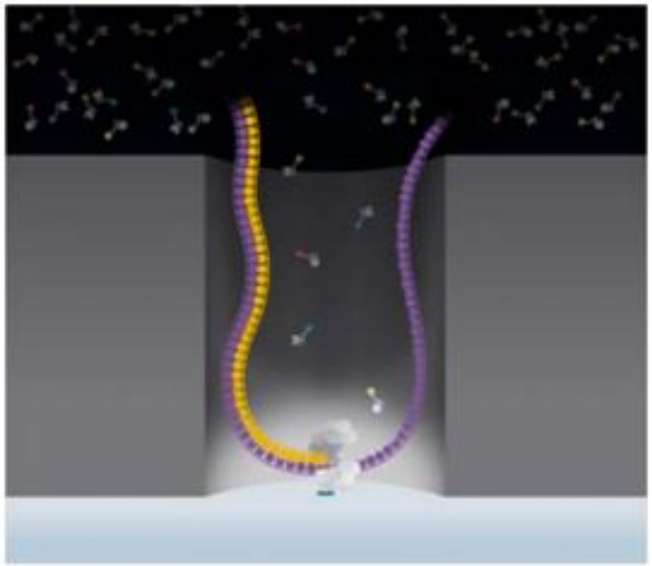
Oxford Nanopore

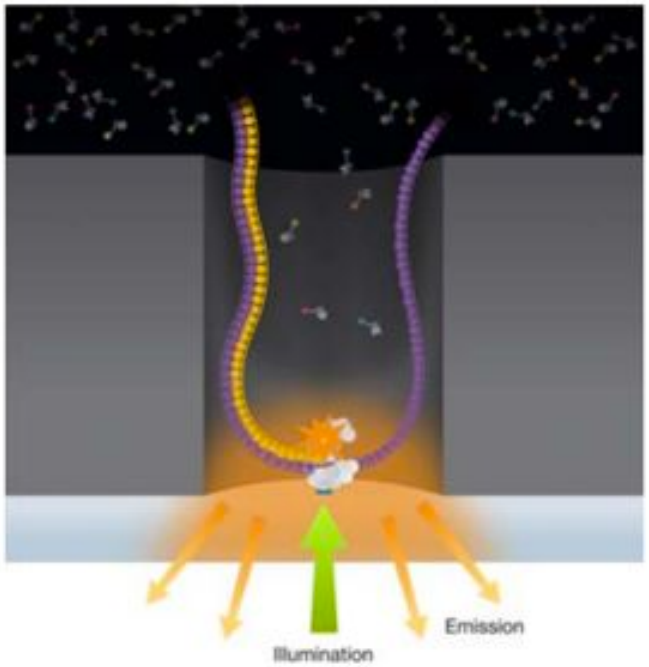


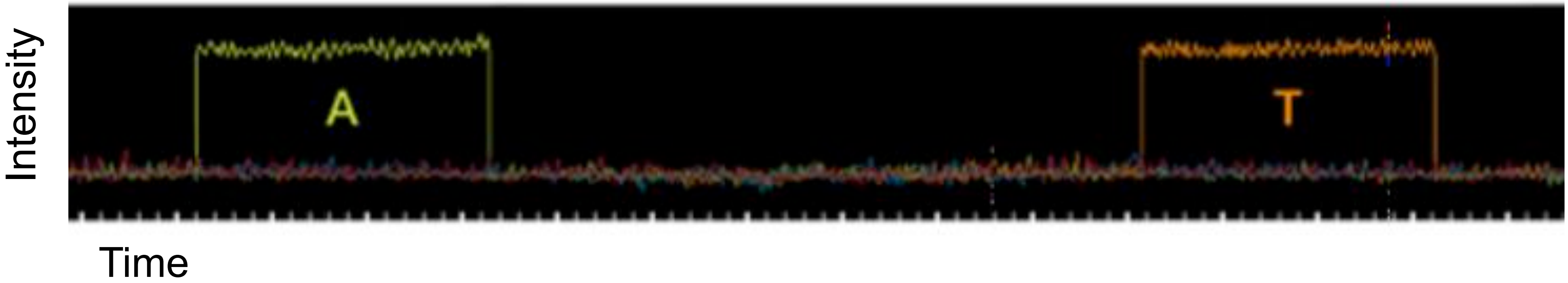
CSHL/ONT

PacBio SMRT Sequencing

Imaging of fluorescently phospholinked labeled nucleotides as they are incorporated by a polymerase anchored to a Zero-Mode Waveguide (ZMW).

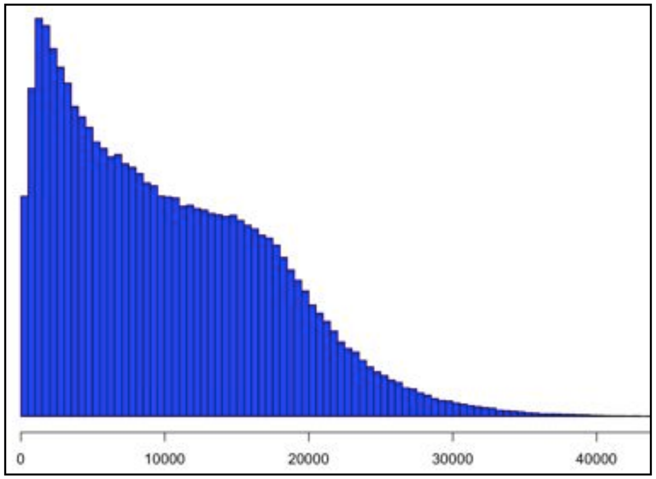






http://www.pacificbiosciences.com/assets/files/pacbio_technology_backgrounder.pdf

SMRT Sequencing Data

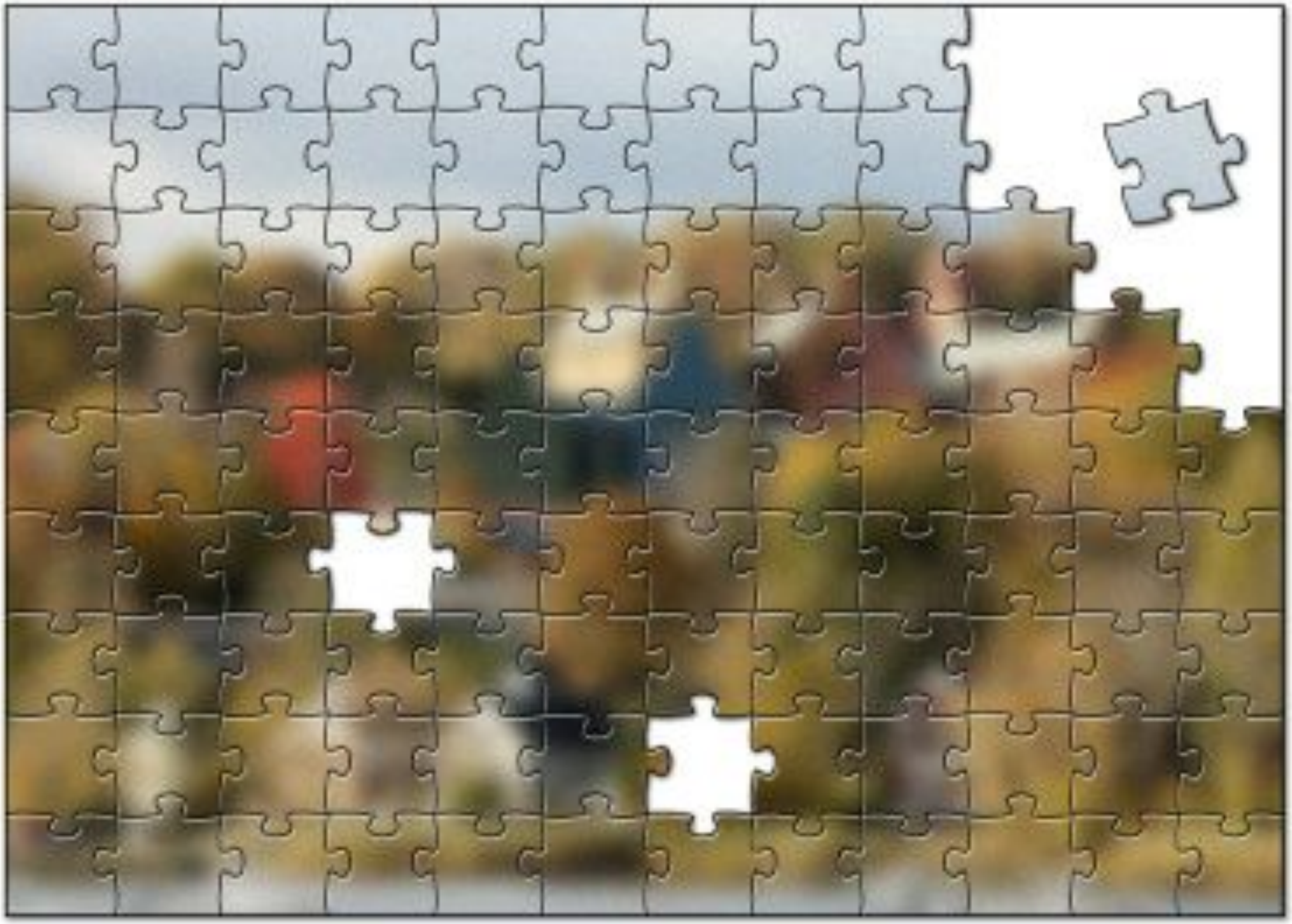


Match	83.7%
Insertions	11.5%
Deletions	3.4%
Mismatch	1.4%

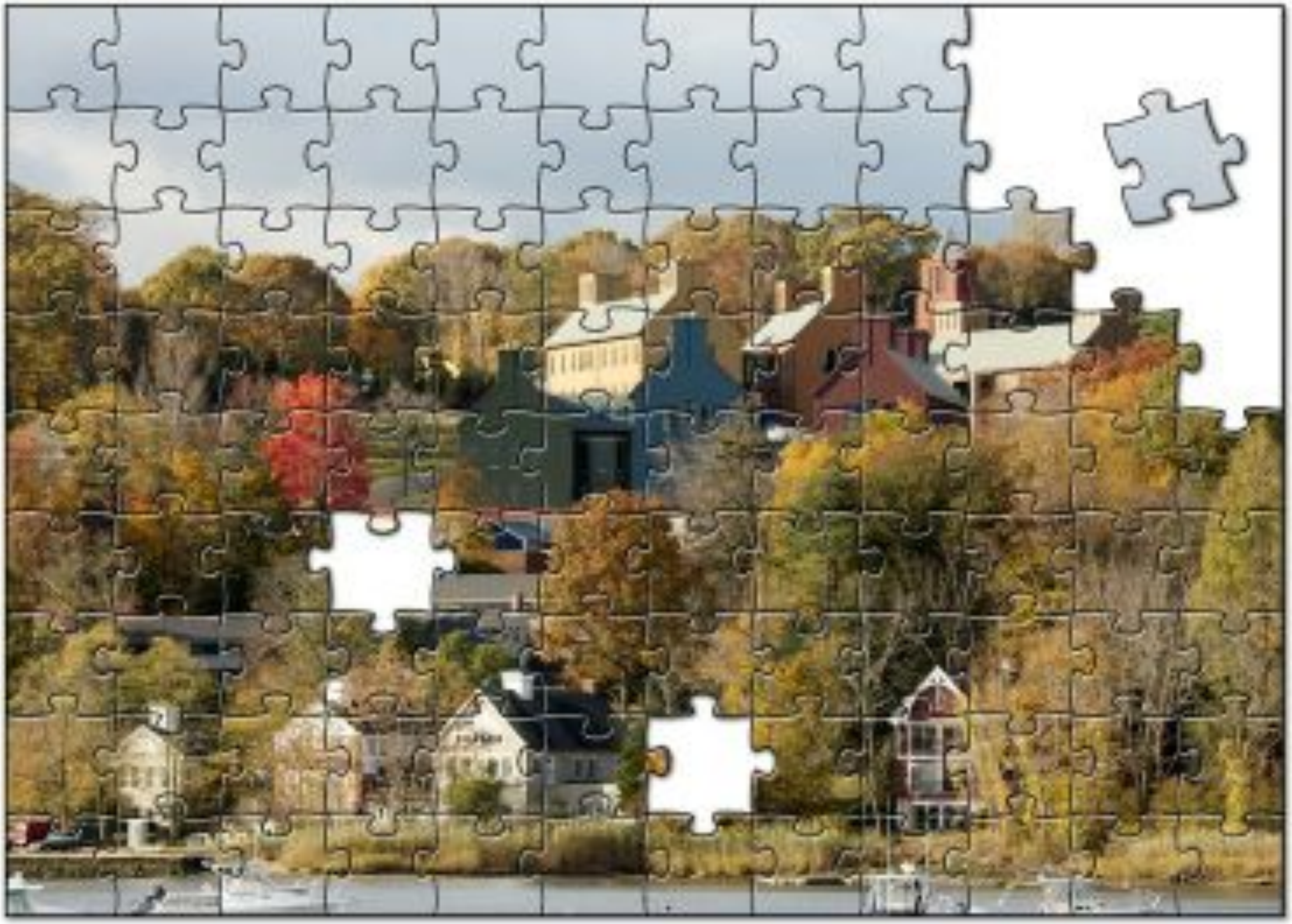
```
TTGTAAGCAGTTGAAAACTATGTGTGGATTTAGAATAAAGAACATGAAAG
||||||||||||||||||||||||| ||||||| |||||||||||| |||
TTGTAAGCAGTTGAAAACTATGTGT-GATTTAG-ATAAAGAACATGGAAG

ATTATAAA-CAGTTGATCCATT-AGAAGA-AAACGCAAAAGGCGGCTAGG
| |||||| ||||||||||||| |||| | |||||| |||||| ||||||
A-TATAAATCAGTTGATCCATTAAGAA-AGAAACGC-AAAGGC-GCTAGG

CAACCTTGAATGTAATCGCACTTGAAGAACAAGATTTTATTCCGCGCCCG
| |||||| |||| ||  ||||||||||||||||||||||||||||||||
C-ACCTTG-ATGT-AT--CACTTGAAGAACAAGATTTTATTCCGCGCCCG

TAACGAATCAAGATTCTGAAAACACAT-ATAACAACCTCCAAAA-CACAA
| ||||||| |||||||||||||| || ||    |||||||||| |||||
T-ACGAATC-AGATTCTGAAAACA-ATGAT----ACCTCCAAAAGCACAA

-AGGAGGGGAAAGGGGGGAATATCT-ATAAAAGATTACAATTAGA-TGA
 ||||||   ||     |||||||| || |||||||||||||| || |||
GAGGAGG---AA-----GAATATCTGAT-AAAGATTACAAATT-GAGTGA

ACT-AATTCACAATA-AATAACACTTTTA-ACAGAATTGAT-GGAA-GTT
||| ||||||||| | ||||||||||||| ||| ||||||| |||| |||
ACTAAATTCACAA-ATAATAACACTTTTAGACAAAATTGATGGGAAGGTT

TCGGAGAGATCCAAAACAATGGGC-ATCGCCTTTGA-GTTAC-AATCAAA
|| ||||||||| ||||||| ||| |||| |||||| ||||| |||||||
TC-GAGAGATCC-AAACAAT-GGCGATCG-CTTTGACGTTACAAATCAAA

ATCCAGTGGAAAATATAATTTATGCAATCCAGGAACTTATTCACAATTAG
||||||| |||||||||  |||||| ||||| ||||||||||||||||||
ATCCAGT-GAAAATATA--TTATGC-ATCCA-GAACTTATTCACAATTAG
```

Sample of 100k reads aligned with BLASR requiring >100bp alignment

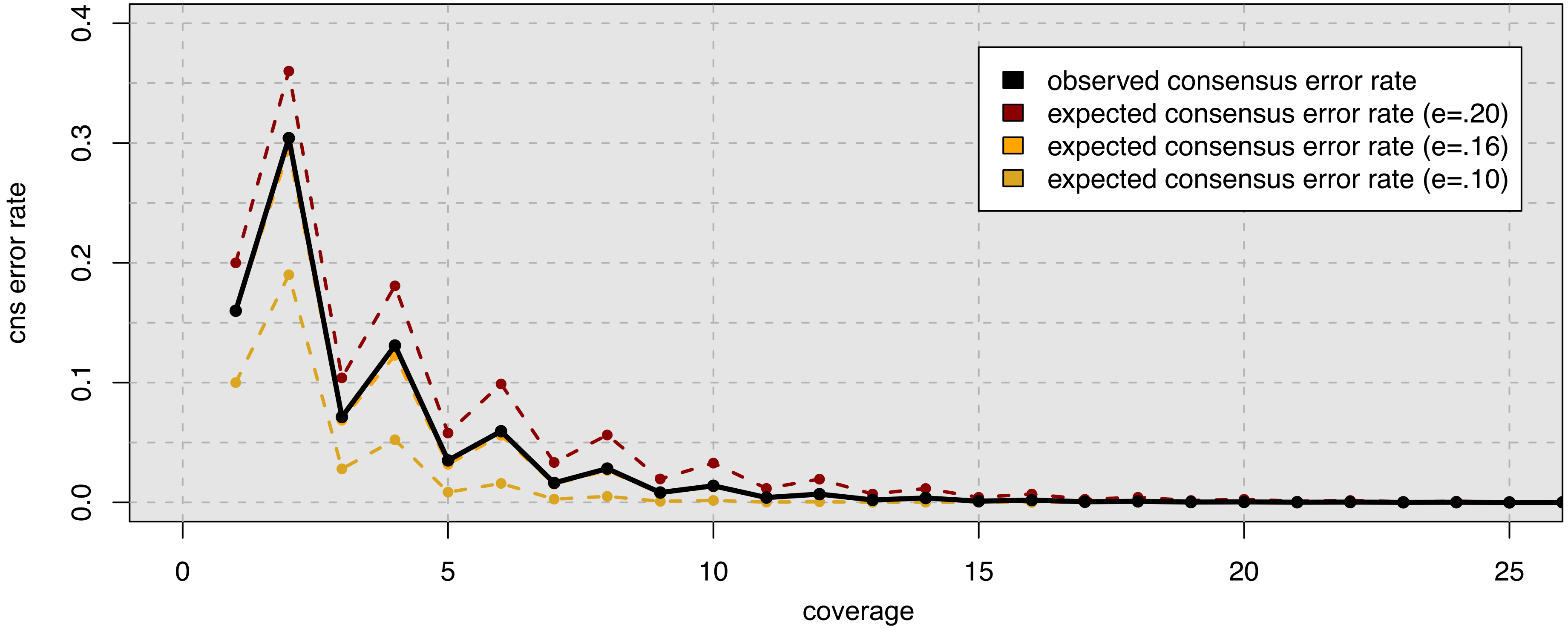
Single Molecule Sequencing



“Corrective Lens” for Sequencing



Consensus Accuracy and Coverage



Coverage can overcome random errors

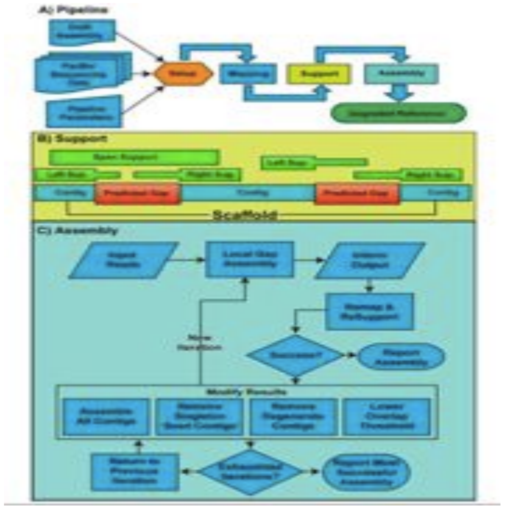
- Dashed: error model from binomial sampling
- Solid: observed accuracy

$$CNS\ Error = \sum_{i=\lceil c/2 \rceil}^{c} \binom{c}{i} (e)^{i} (1-e)^{n-i}$$

Koren, Schatz, *et al* (2012)
Nature Biotechnology. 30:693–700

PacBio Assembly Algorithms

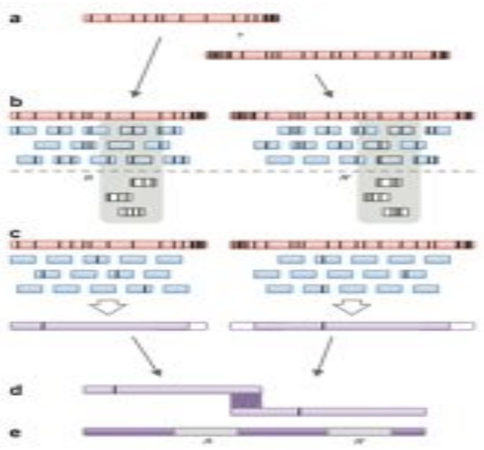
PBJelly



Gap Filling and Assembly Upgrade

English *et al* (2012)
PLOS One. 7(11): e47768

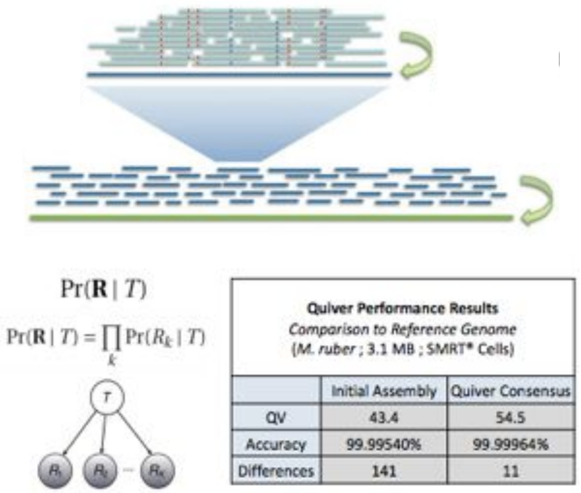
PacBioToCA & ECTools



Hybrid/PB-only Error Correction

Koren, Schatz, *et al* (2012)
Nature Biotechnology. 30:693–700

HGAP & Quiver



Quiver Performance Results
Comparison to Reference Genome
(*M. ruber* ; 3.1 MB ; SMRT® Cells)

	Initial Assembly	Quiver Consensus
QV	43.4	54.5
Accuracy	99.99540%	99.99964%
Differences	141	11

PB-only Correction & Polishing

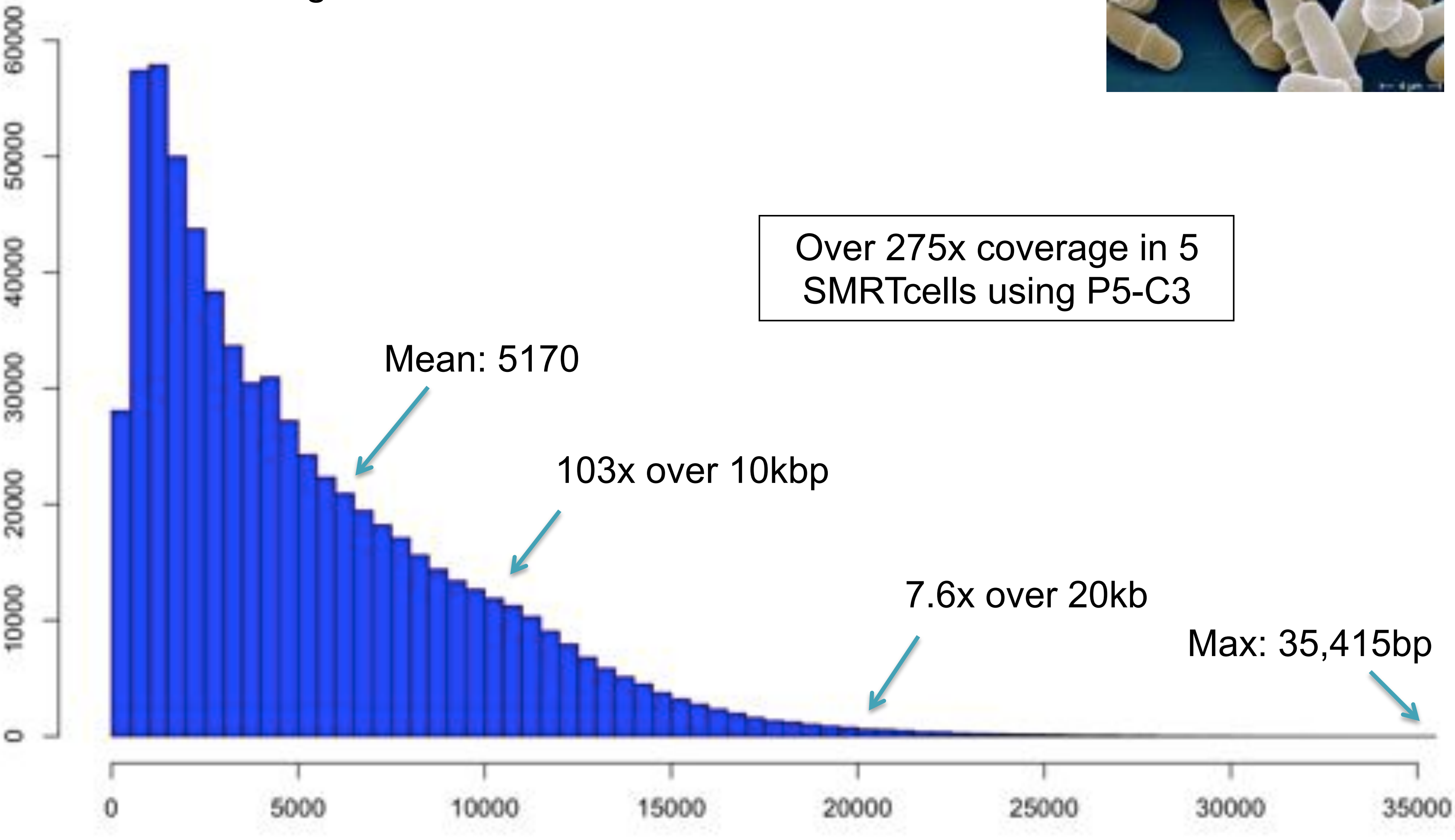
Chin *et al* (2013)
Nature Methods. 10:563–569

< 5x PacBio Coverage > 50x

S. pombe dg21

PacBio RS II sequencing at CSHL

- Size selection using an 7 Kb elution window on a BluePippin™ device from Sage Science



Over 275x coverage in 5 SMRTcells using P5-C3

Mean: 5170

103x over 10kbp

7.6x over 20kb

Max: 35,415bp

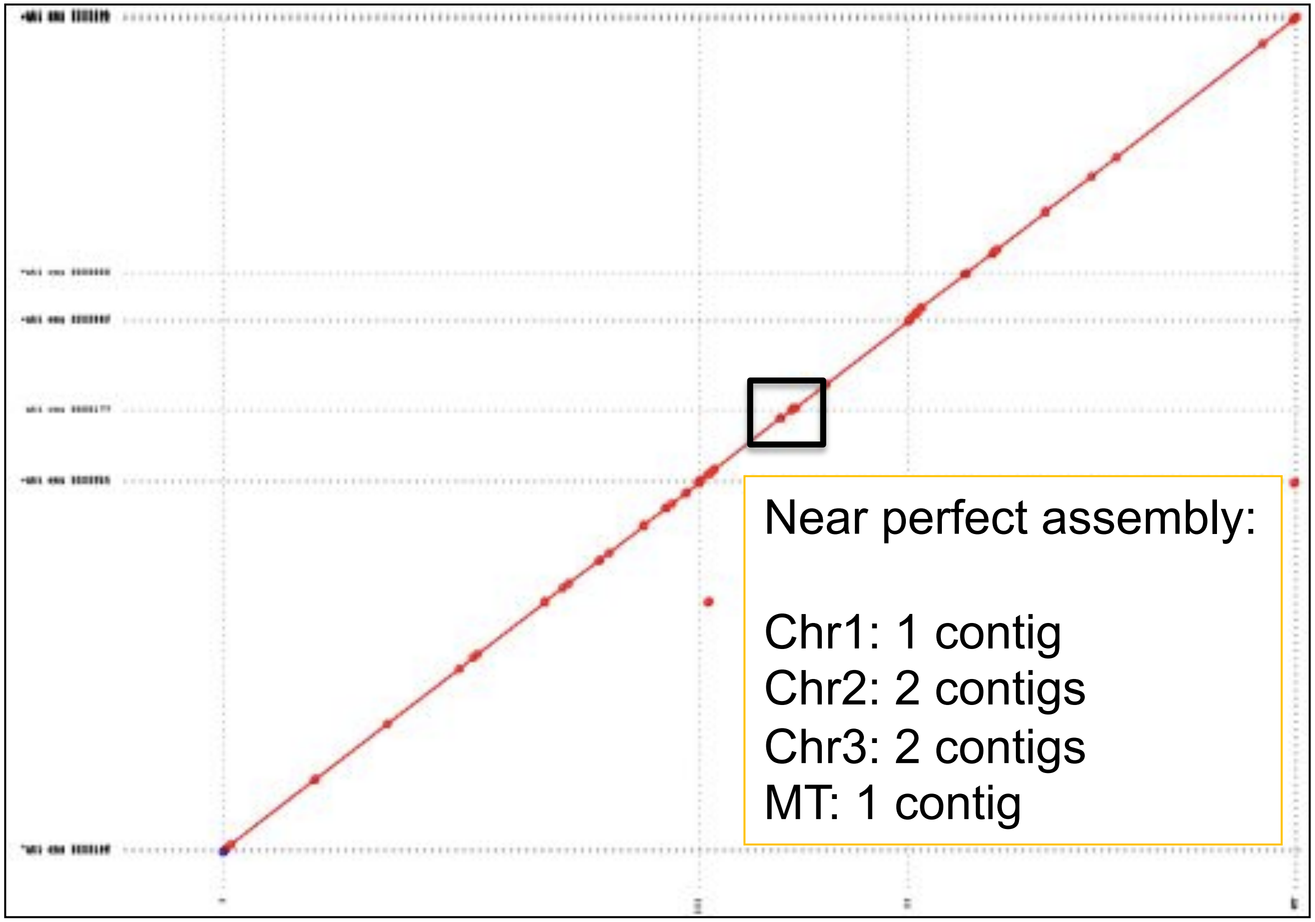
S. pombe dg21

ASM294 Reference sequence

- 12.6Mbp; 3 chromo + mitochondria; N50: 4.53Mbp

PacBio assembly using HGAP + Celera Assembler

- 12.7Mbp; 13 non-redundant contigs; N50: 3.83Mbp; >99.98% id



Near perfect assembly:

Chr1: 1 contig
Chr2: 2 contigs
Chr3: 2 contigs
MT: 1 contig

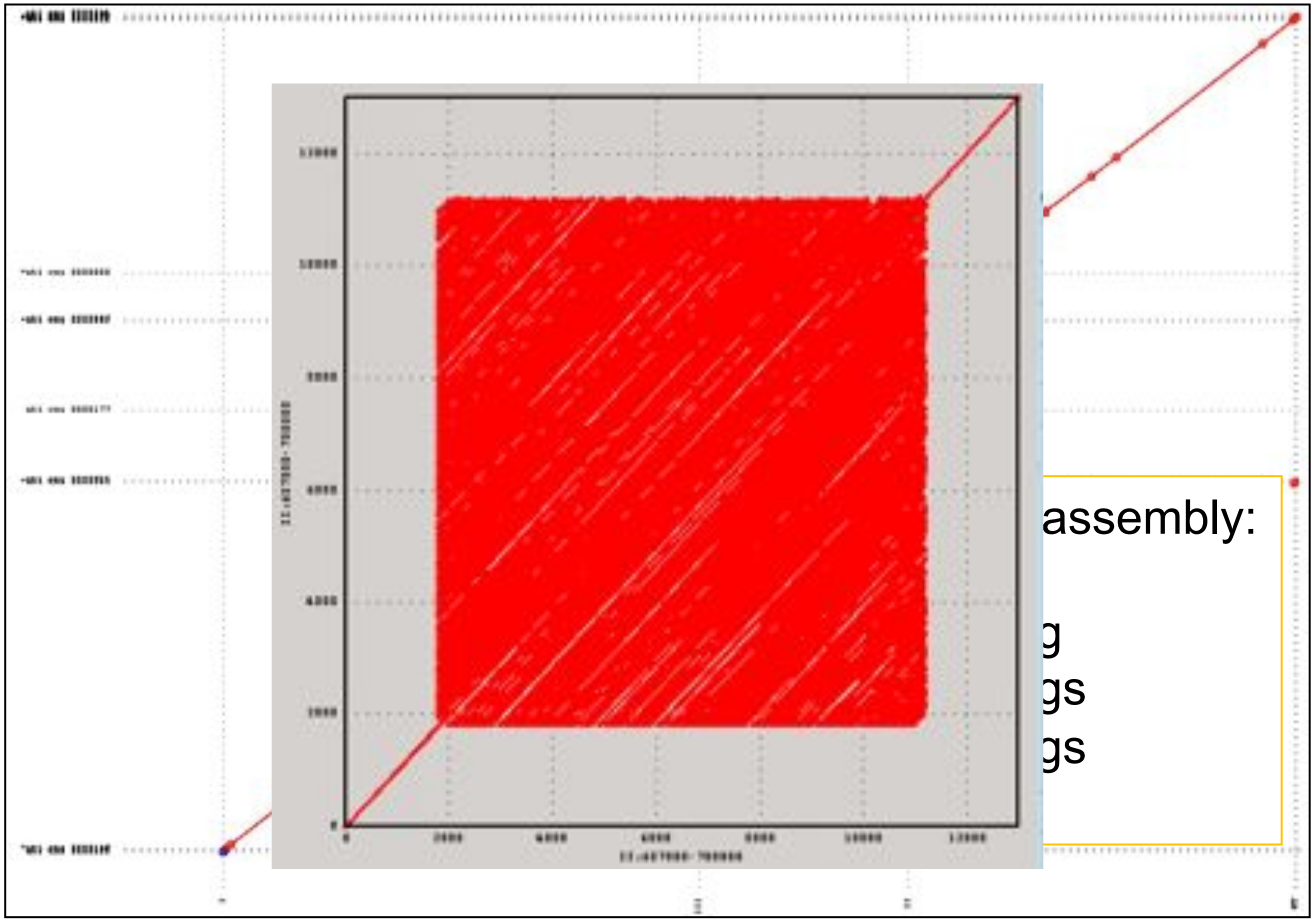
S. pombe dg21

ASM294 Reference sequence

- 12.6Mbp; 3 chromo + mitochondria; N50: 4.53Mbp

PacBio assembly using HGAP + Celera Assembler

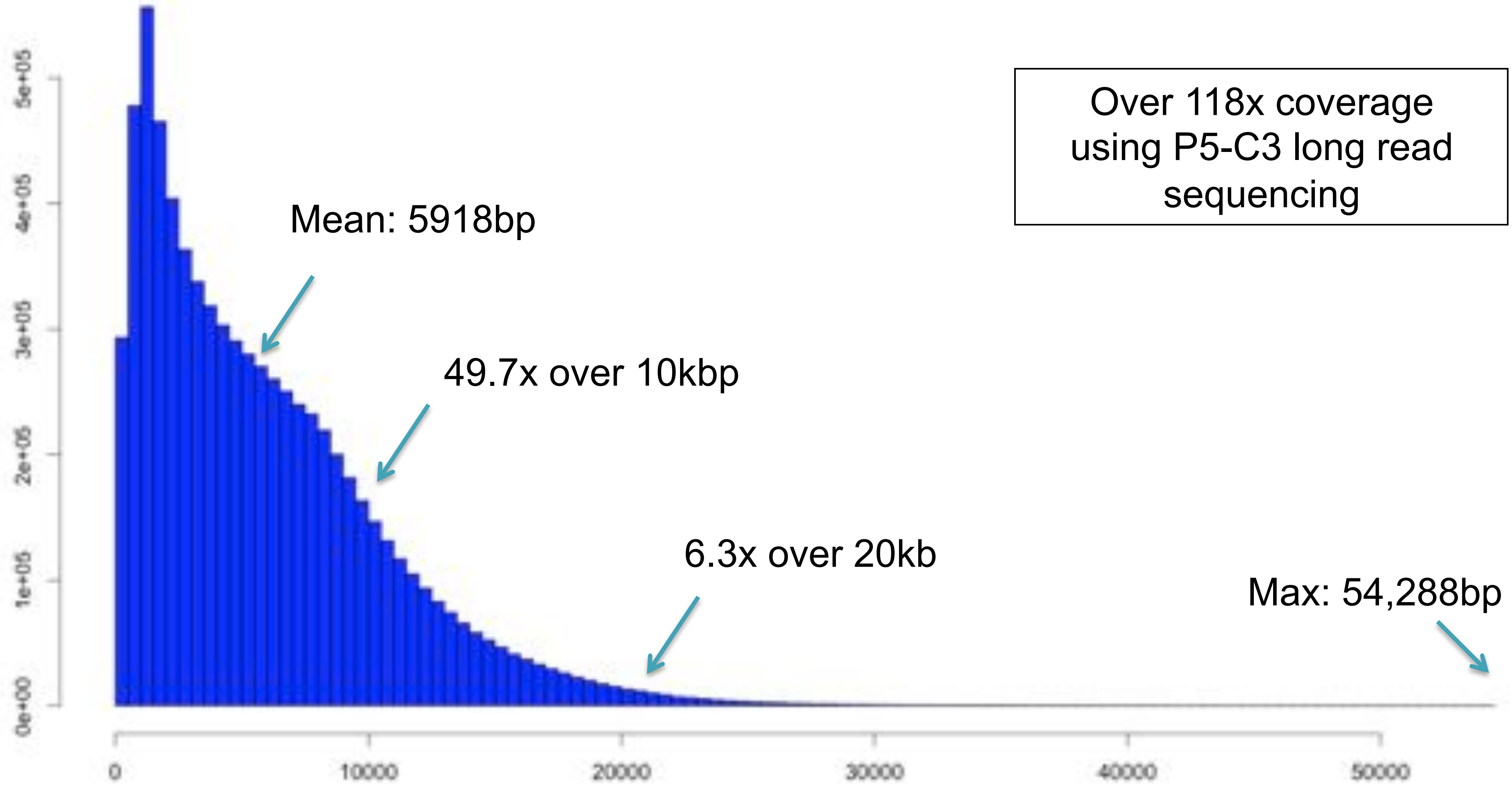
- 12.7Mbp; 13 non-redundant contigs; N50: 3.83Mbp; >99.98% id



O. sativa pv Indica (IR64)

PacBio RS II sequencing at PacBio

- Size selection using an 10 Kb elution window on a BluePippin™ device from Sage Science



Over 118x coverage using P5-C3 long read sequencing

Mean: 5918bp

49.7x over 10kbp

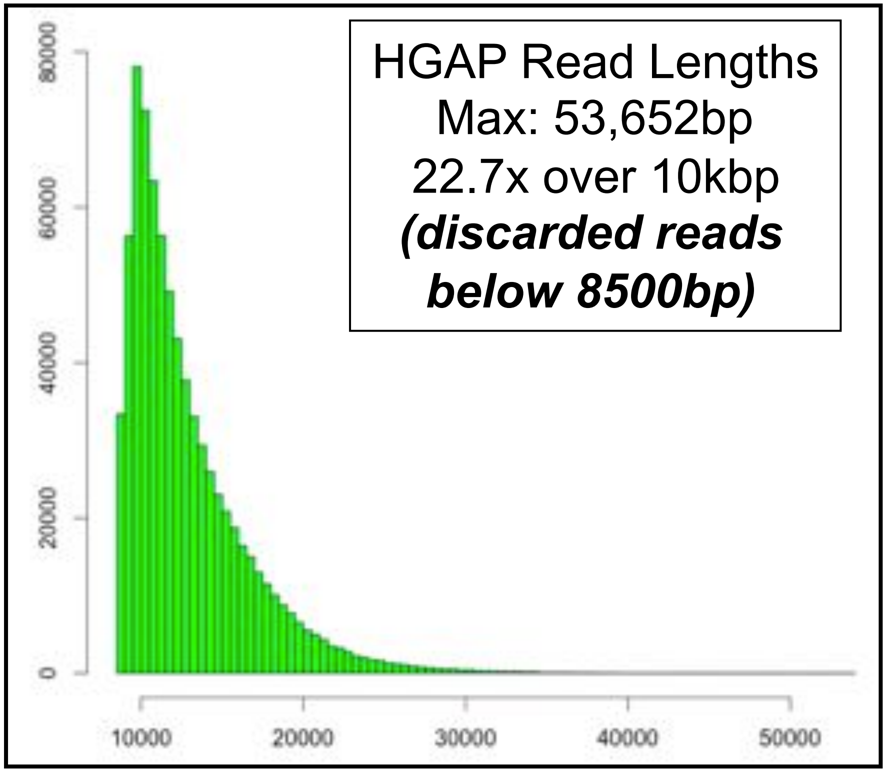
6.3x over 20kb

Max: 54,288bp

O. sativa pv Indica (IR64)

Genome size: ~370 Mb
Chromosome N50: ~29.7 Mbp

Assembly	Contig NG50
MiSeq Fragments 25x 456bp (3 runs 2x300 @ 450 FLASH)	19 kbp
“ALLPATHS-recipe” 50x 2x100bp @ 180 36x 2x50bp @ 2100 51x 2x50bp @ 4800	18 kbp
HGAP 22.7x @ 10kbp	4.0 Mbp
Nipponbare BAC-by-BAC Assembly	5.1 Mbp



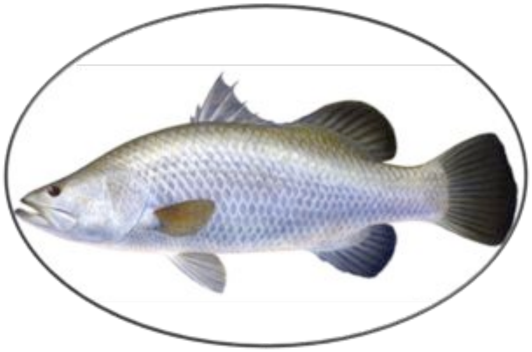
HGAP Read Lengths
Max: 53,652bp
22.7x over 10kbp
(discarded reads below 8500bp)

Current Collaborations

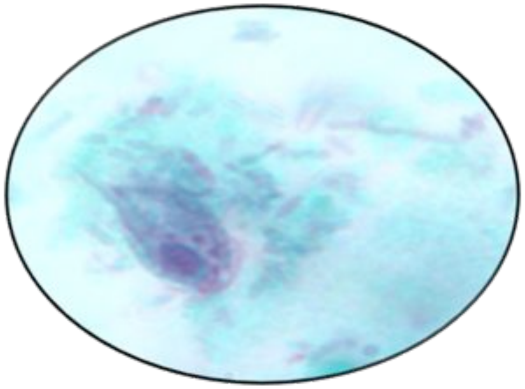
M. ligano
Hannon

Pinapple
UIUC

Human
CSHL/OICR/PacBio

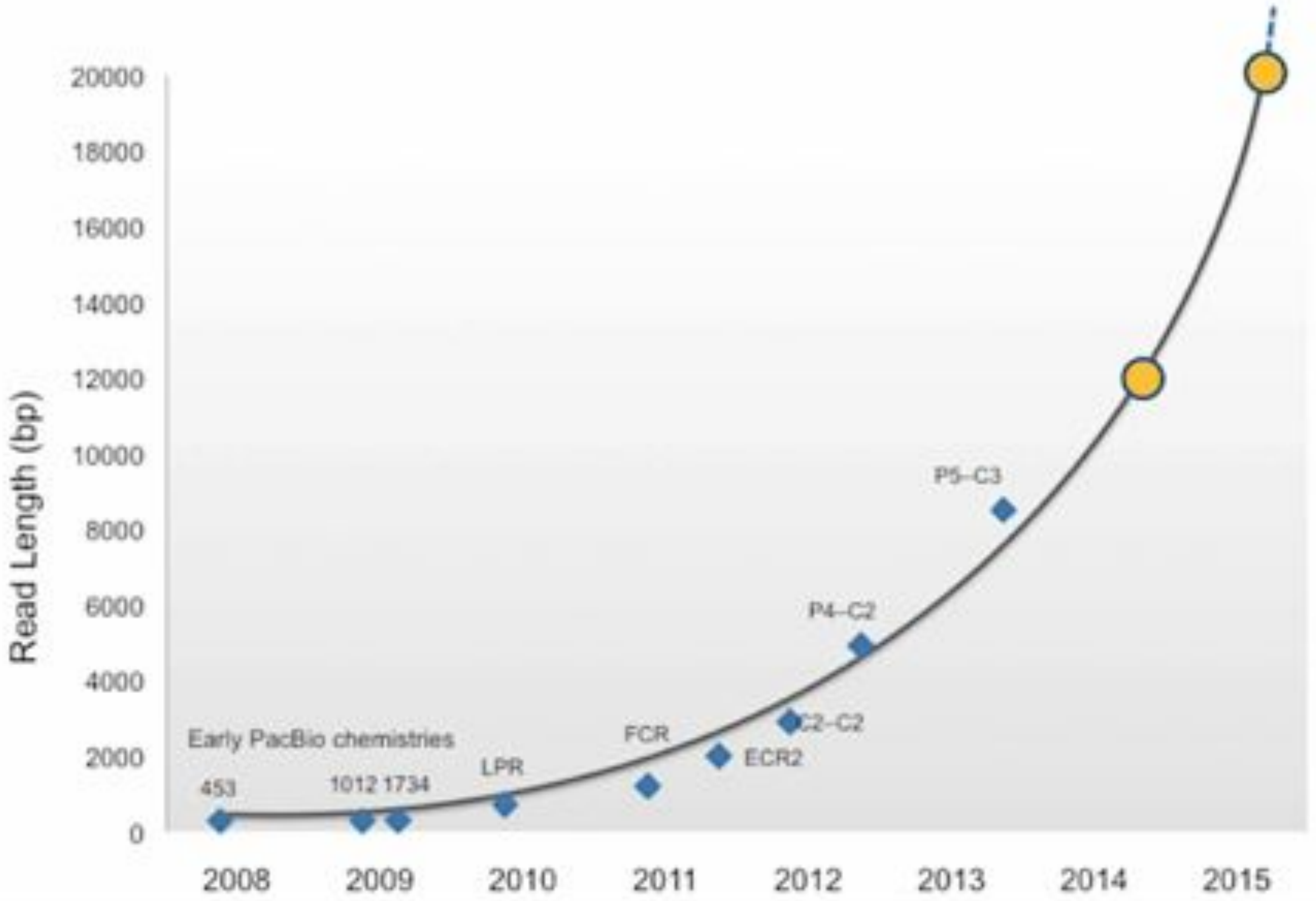


Asian Sea Bass
Temasek Life Sciences



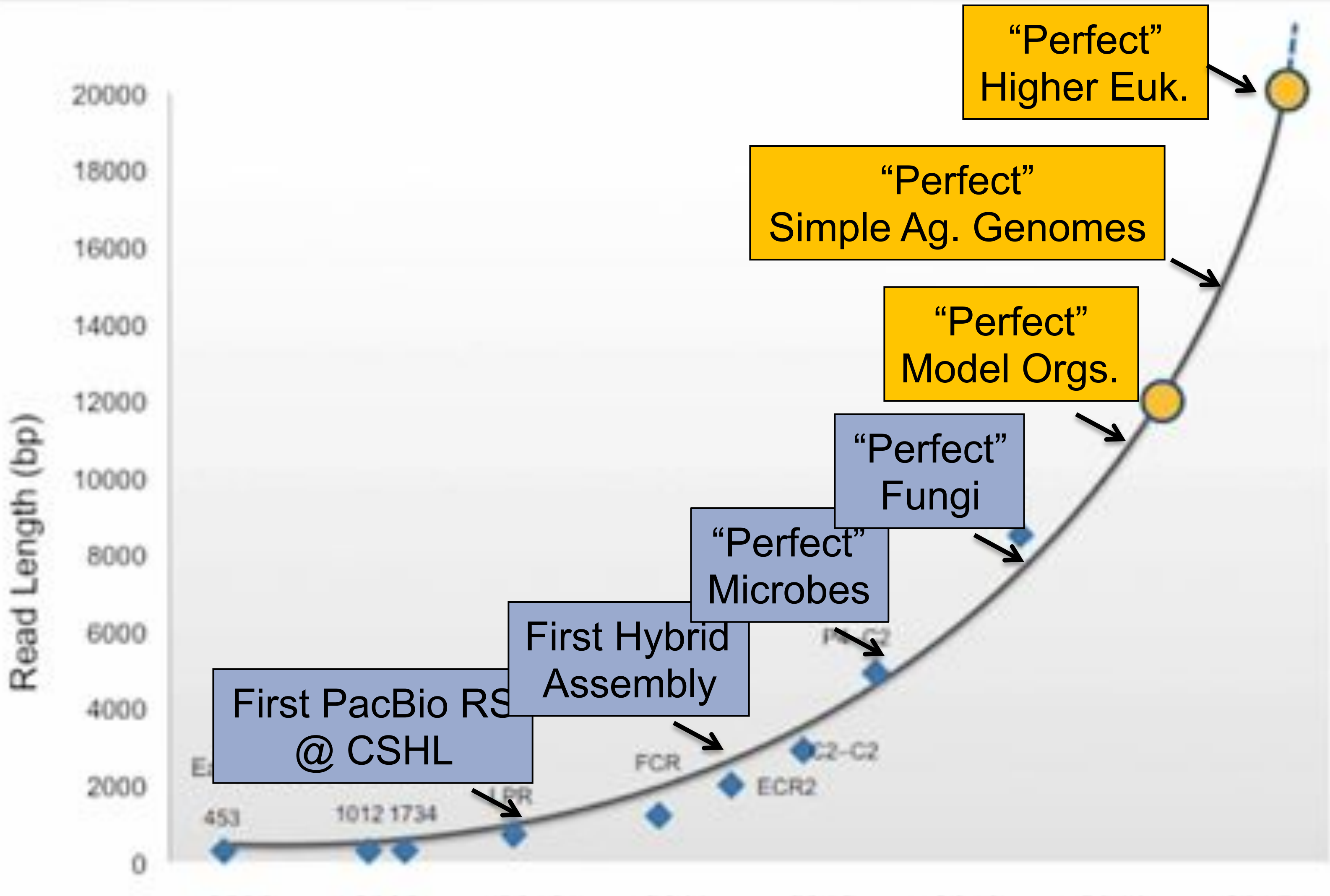
P. hominis
NYU

PacBio® Advances in Read Length



Advances in Assembly

“Perfect” Human Assembly



“Perfect” Higher Euk.

“Perfect” Simple Ag. Genomes

“Perfect” Model Orgs.

“Perfect” Fungi

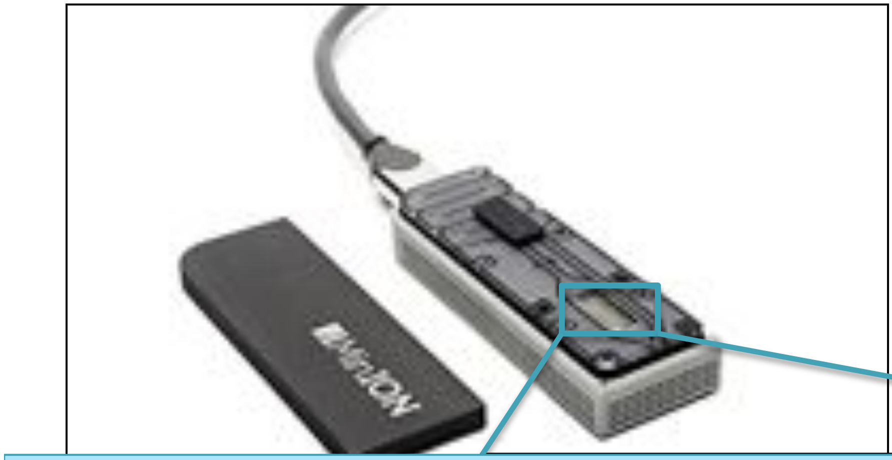
“Perfect” Microbes

First Hybrid Assembly

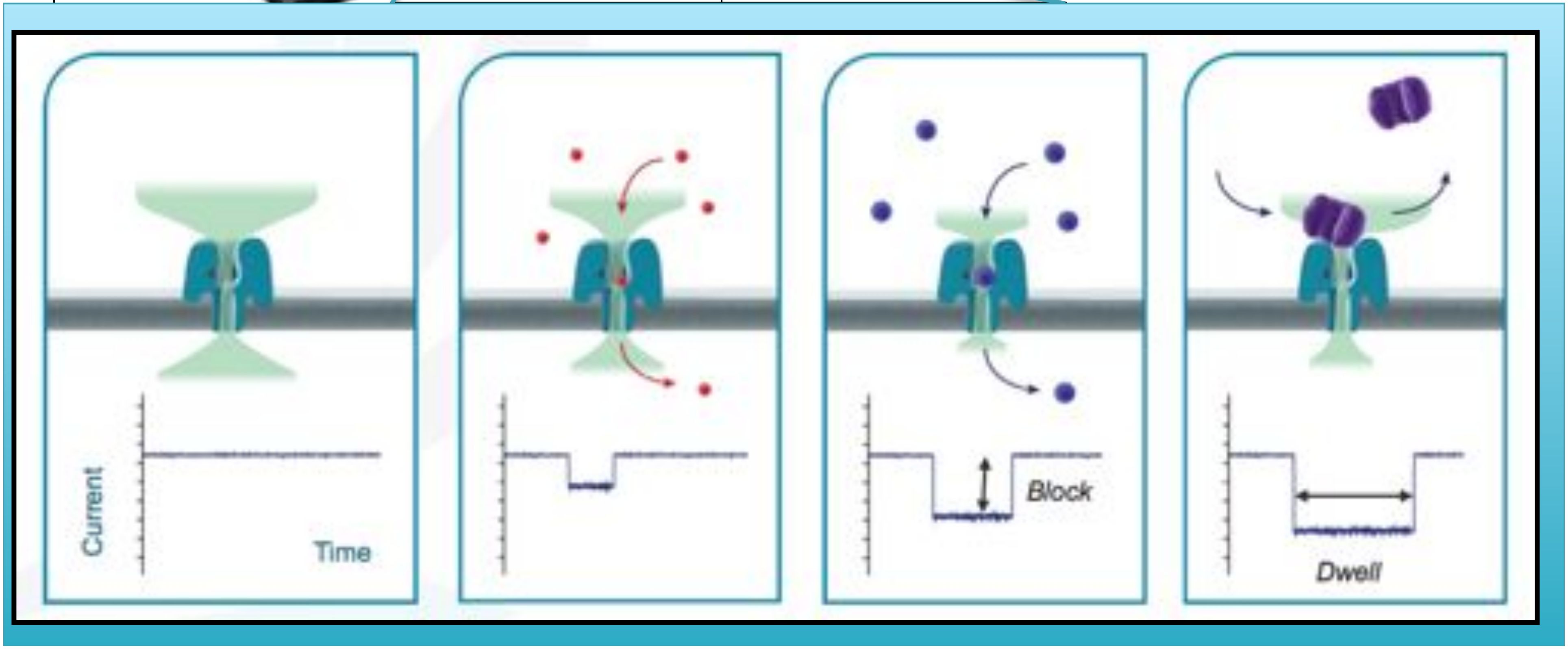
First PacBio RS @ CSHL

Error correction and assembly complexity of single molecule sequencing reads.
Lee, H*, Gurtowski, J*, Yoo, S, Marcus, S, McCombie, WR, Schatz, MC
http://www.biorxiv.org/content/early/2014/06/18/006395

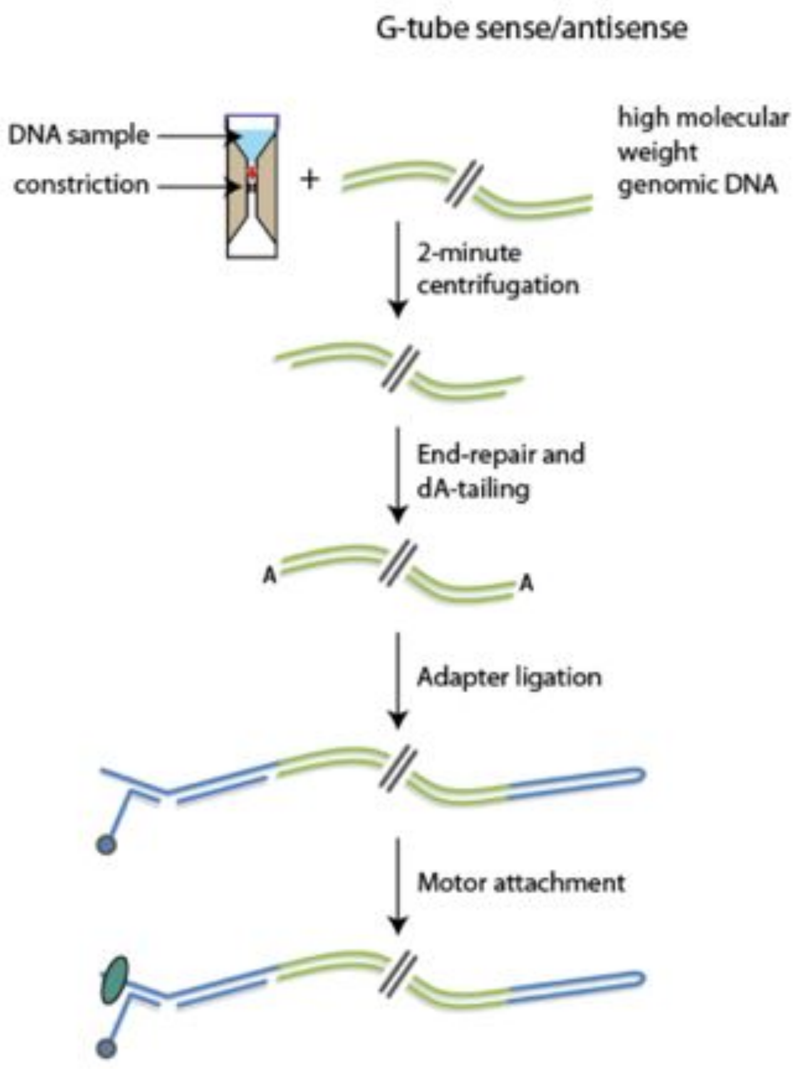
Oxford Nanopore MinION

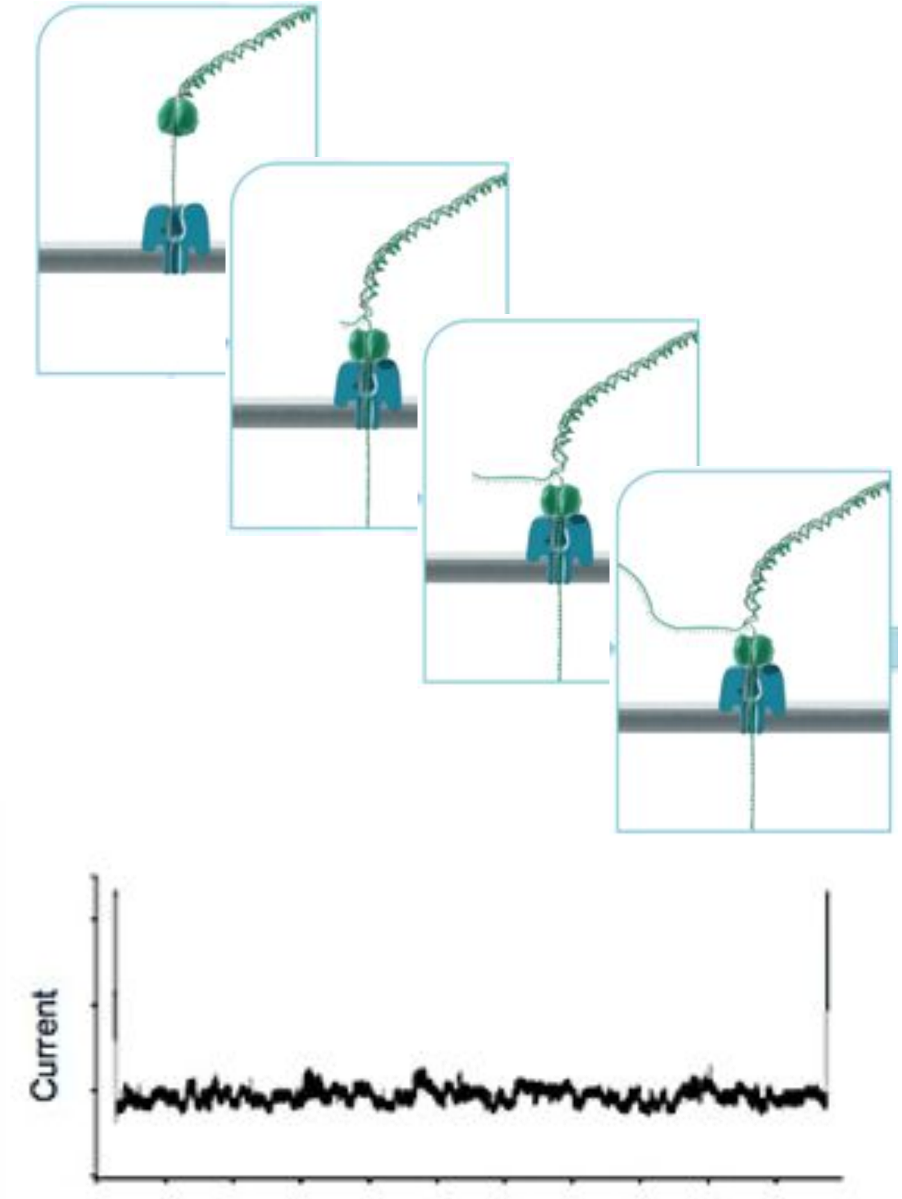


- Thumb drive sized sequencer powered over USB
- Capacity for 512 reads at once
- Senses DNA by measuring changes to ion flow

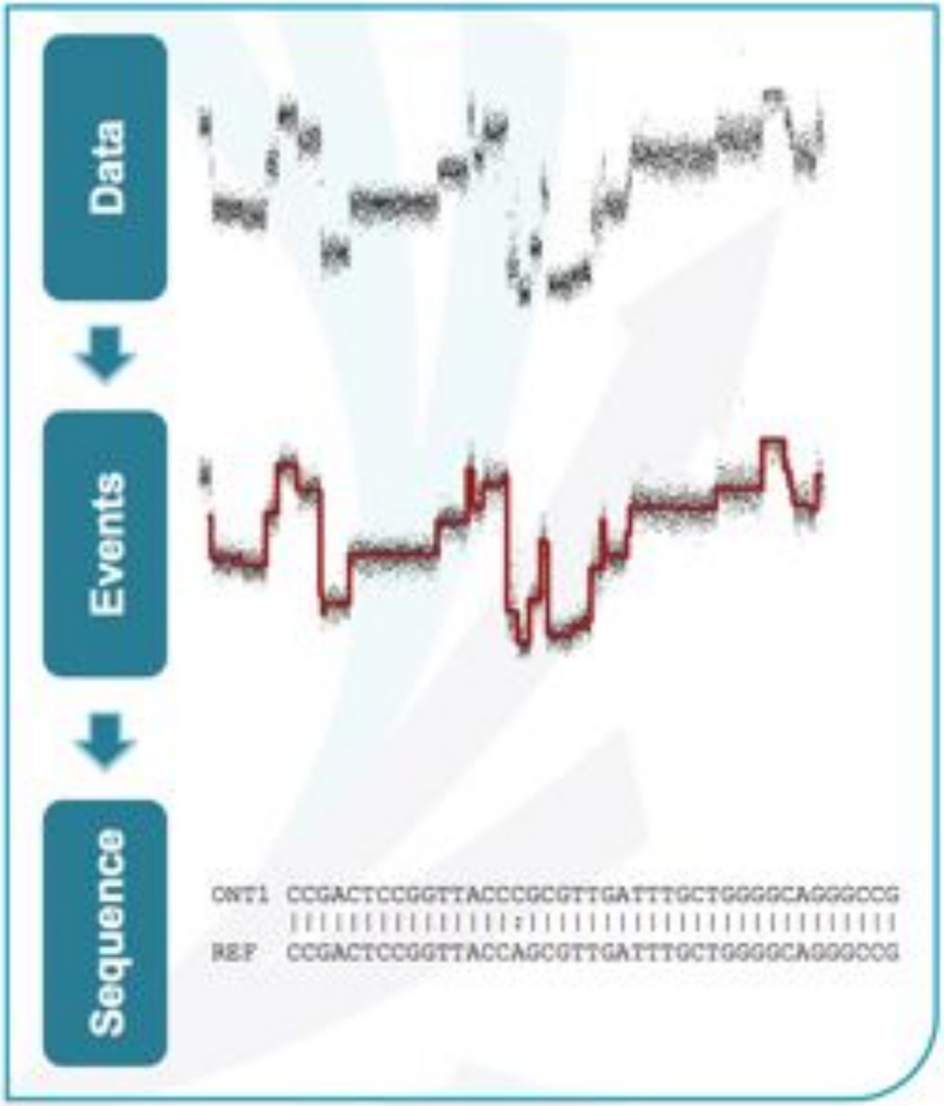


Nanopore Sequencing



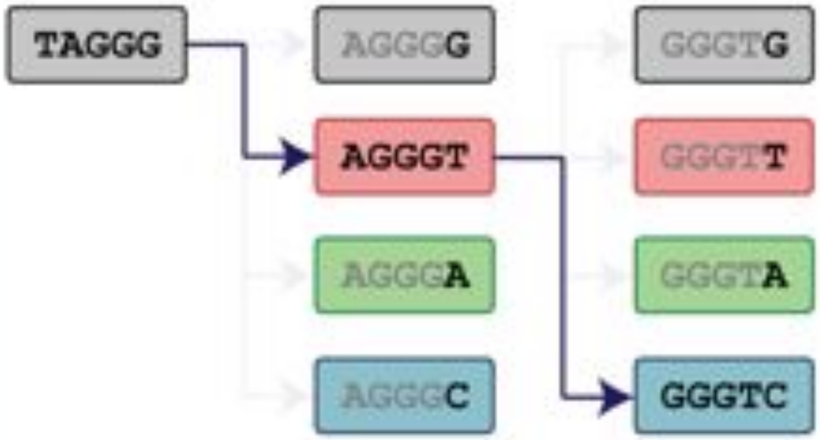


Nanopore Basecalling



```
ONT1 CCGACTCCGGTTACCCGCGTTGATTTGCTGGGGCAGGGCCG
     |||||||||||||||:|||||||||||||||||||||||||
REF  CCGACTCCGGTTACCAGCGTTGATTTGCTGGGGCAGGGCCG
```

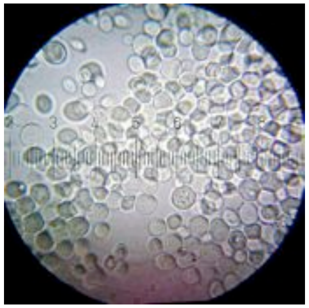
- Hidden Markov model
- Only four options per transition
- Pore type = distinct kmer length



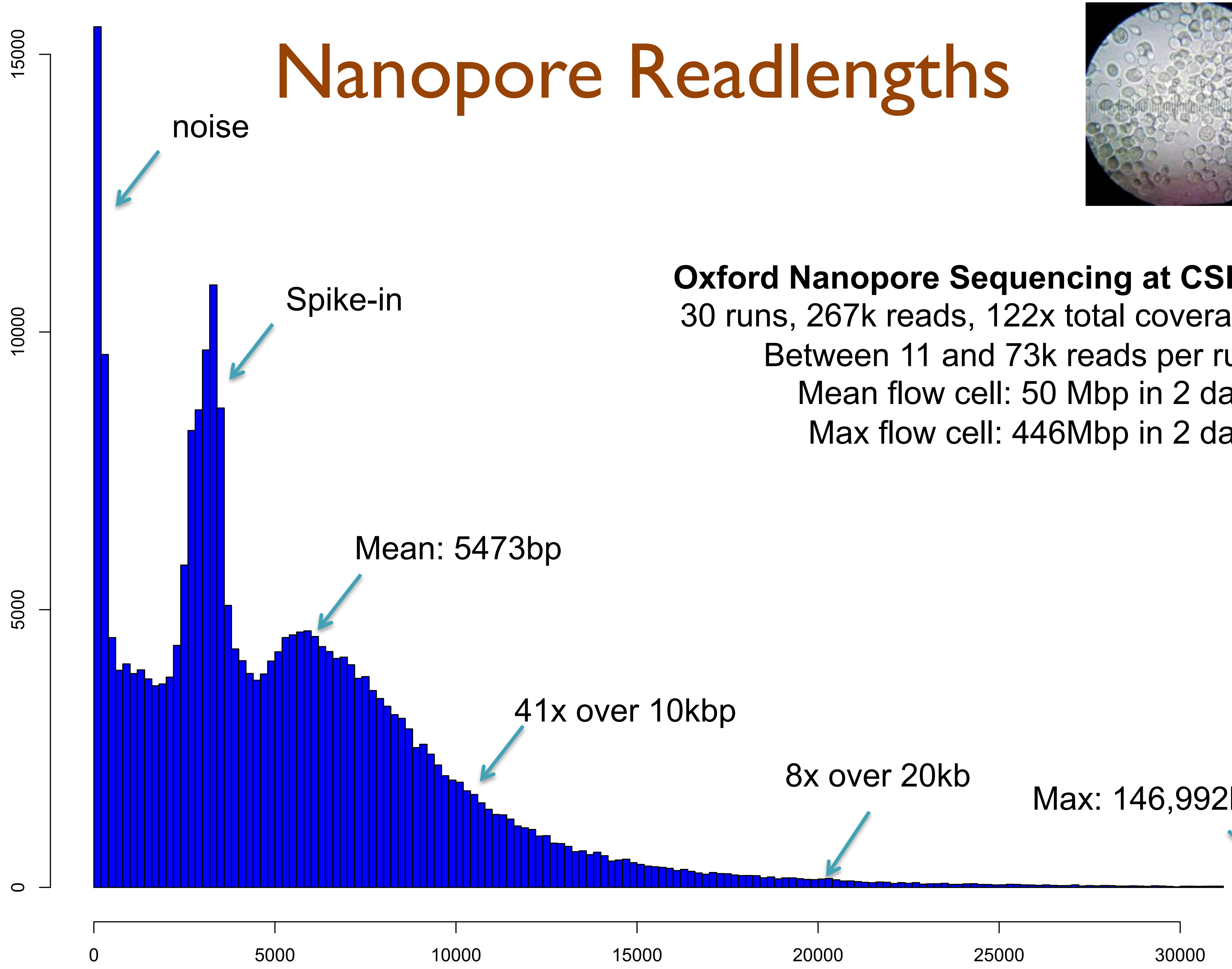
- Form probabilistic path through measured states currents and transitions
  - e.g. Viterbi algorithm

Basecalling currently performed at Amazon with frequent updates to algorithm

Nanopore Readlengths



Oxford Nanopore Sequencing at CSHL
30 runs, 267k reads, 122x total coverage
Between 11 and 73k reads per run!
Mean flow cell: 50 Mbp in 2 days
Max flow cell: 446Mbp in 2 days



noise

Spike-in

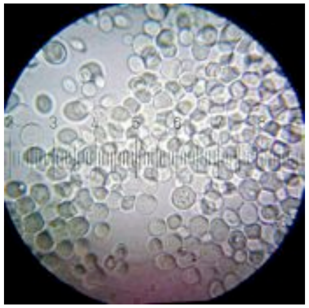
Mean: 5473bp

41x over 10kbp

8x over 20kb

Max: 146,992bp

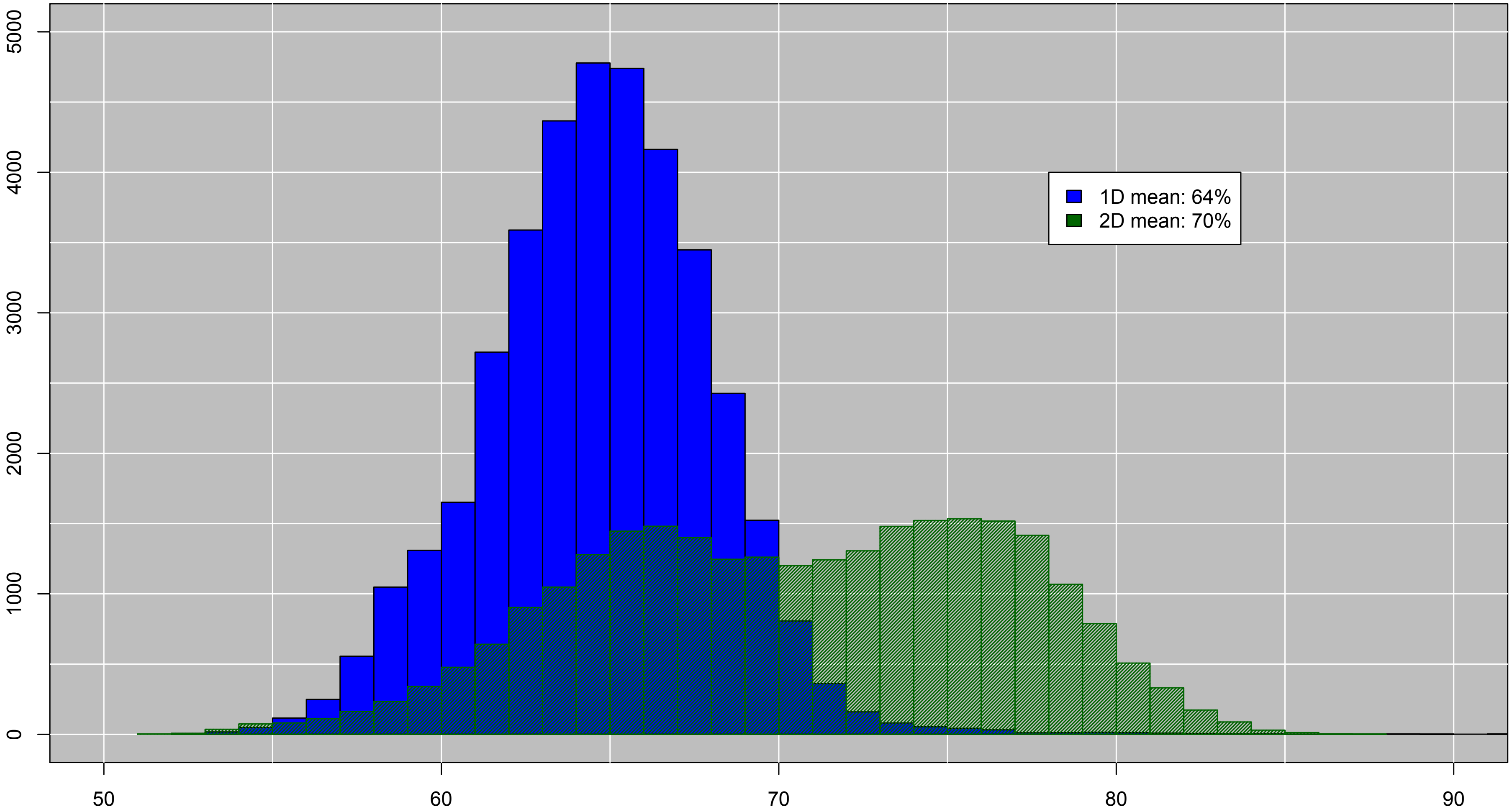
Nanopore Accuracy



Alignment Quality (BLASTN)

Of reads that align, average ~64% identity

“2D base-calling” improves to ~70% identity



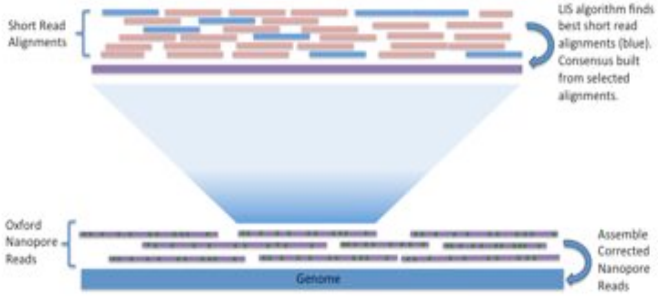
NanoCorr: Nanopore-Illumina Hybrid Error Correction

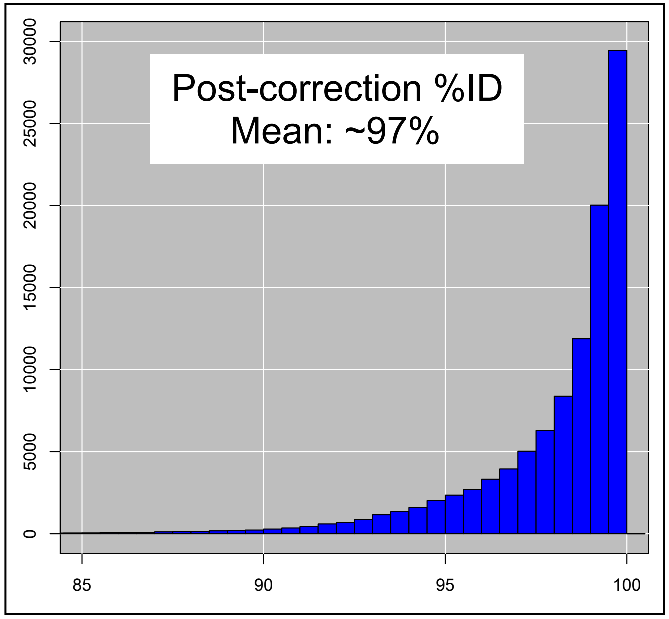
https://github.com/jgurtowski/nanocorr

1. BLAST Miseq reads to all raw Oxford Nanopore reads

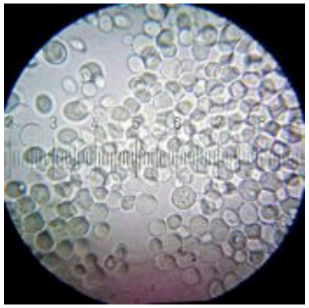
2. Select non-repetitive alignments
   - ○ First pass scans to remove "contained" alignments
   - ○ Second pass uses Dynamic Programming (LIS) to select set of high-identity alignments with minimal overlaps

3. Compute consensus of each Oxford Nanopore read
   - ○ Currently using Pacbio's pbdagcon


Short Read Alignments
LIS algorithm finds best short read alignments (blue). Consensus built from selected alignments.
Oxford Nanopore Reads
Genome
Assemble Corrected Nanopore Reads



Long Read Assembly



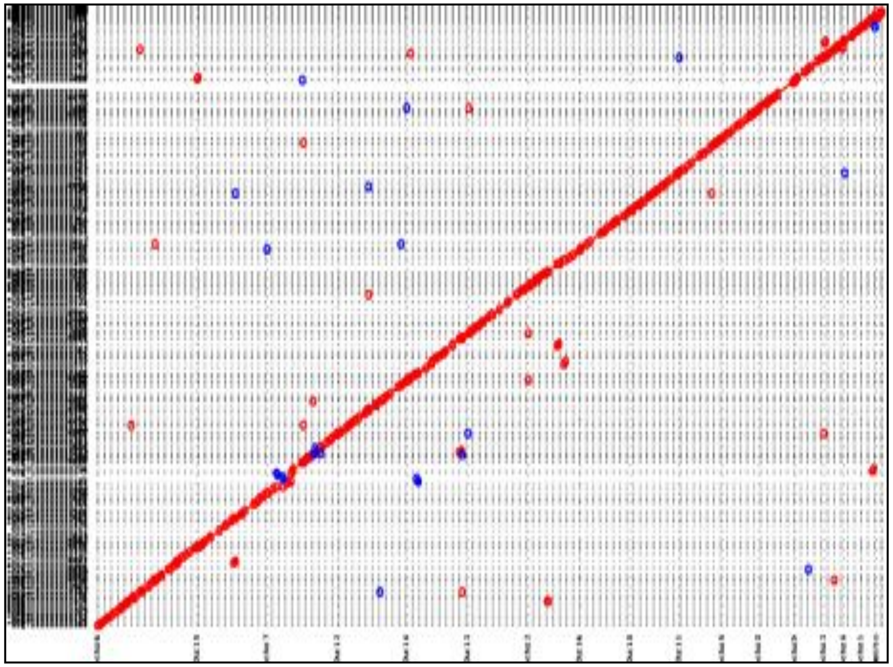
S288C Reference sequence

- 12.1Mbp; 16 chromo + mitochondria; N50: 924kbp

Illumina MiSeq

30x, 300bp PE (Flashed)

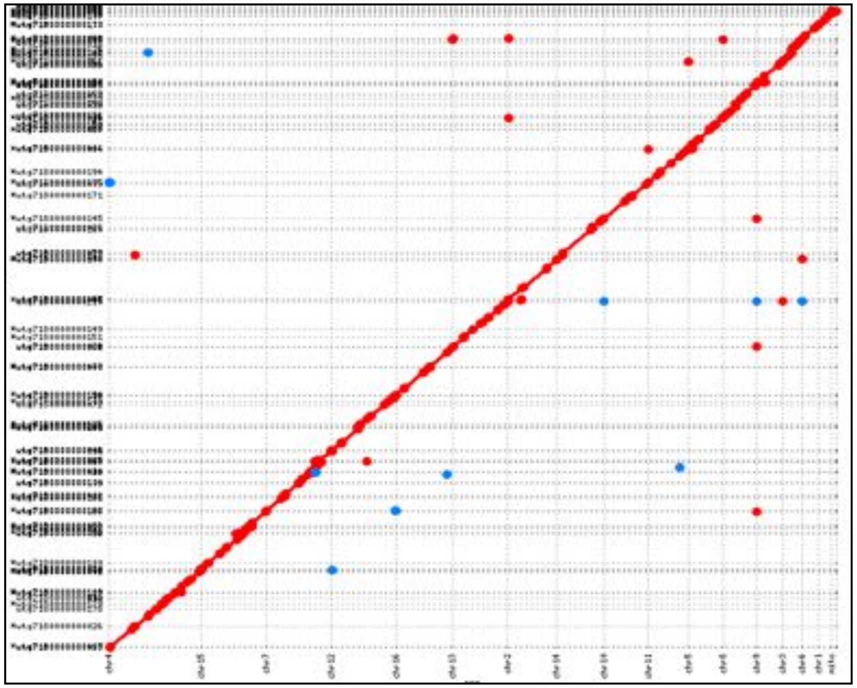
- 6953 non-redundant contigs
- N50:59kbp >99.9% id



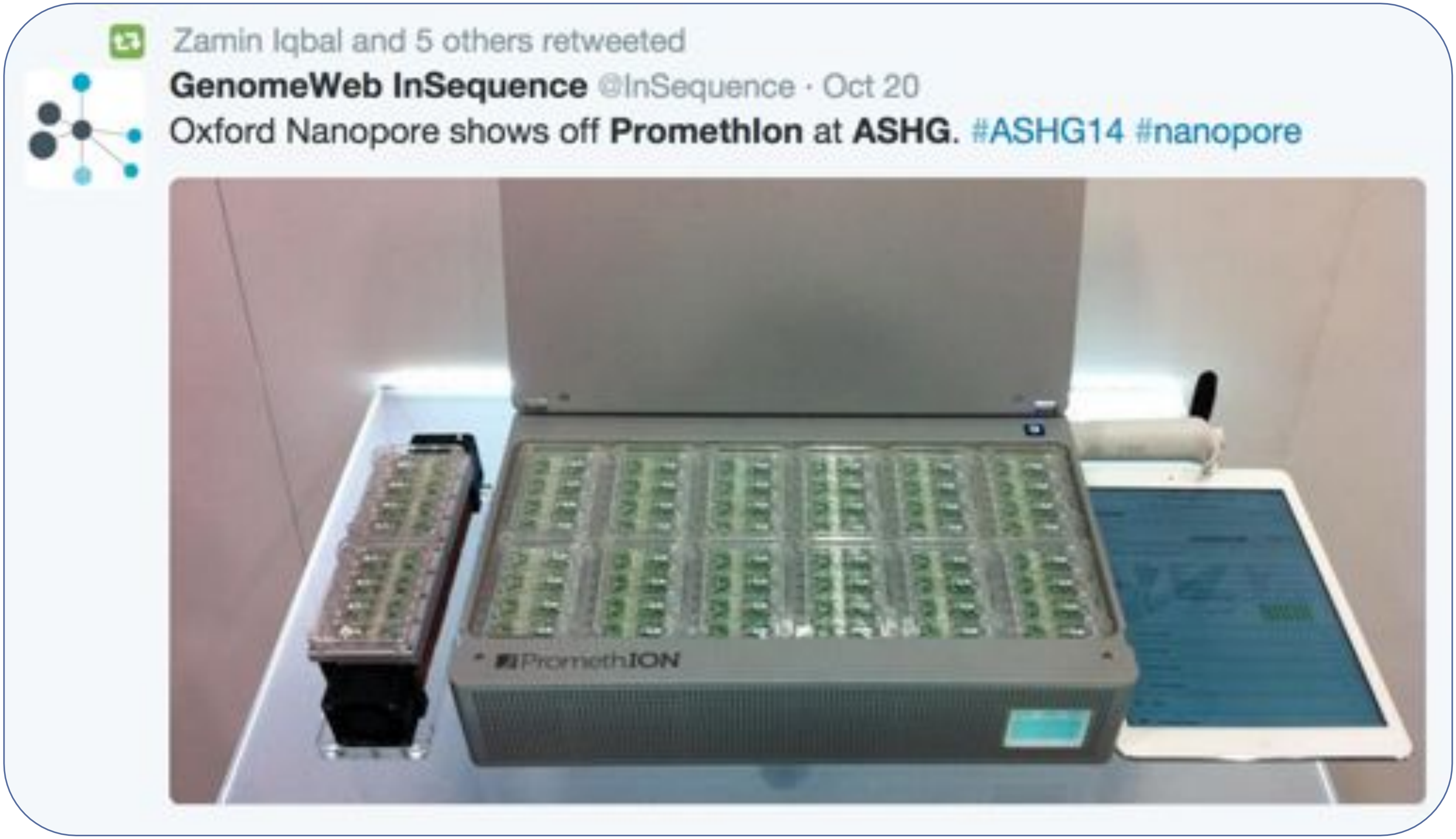
Oxford Nanopore

NanoCorr + Celera Assembler

- 214 non-redundant contigs
- N50: 472kbp >99.78% id



Genomic Futures?

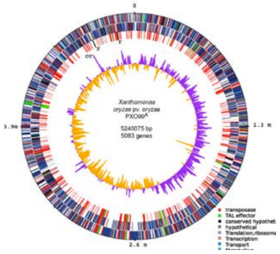


Zamin Iqbal and 5 others retweeted

GenomeWeb InSequence @InSequence · Oct 20

Oxford Nanopore shows off **PromethIon** at **ASHG**. #ASHG14 #nanopore

Assembly Summary



Assembly quality depends on

1. ***Coverage***: low coverage is mathematically hopeless
2. ***Repeat composition***: high repeat content is challenging
3. ***Read length***: longer reads help resolve repeats
4. ***Error rate***: errors reduce coverage, obscure true overlaps

- Assembly is a hierarchical, starting from individual reads, build high confidence contigs/unitigs, incorporate the mates to build scaffolds
  - Extensive error correction is the key to getting the best assembly possible from a given data set

- Watch out for collapsed repeats & other misassemblies
  - Globally/Locally reassemble data from scratch with better parameters & stitch the 2 assemblies together

What should we expect from an assembly?

Analysis of dozens of genomes from across the tree of life with real and simulated data

Summary & Recommendations

< 100 Mbp:	HGAP/PacBio2CA @ 100x PB C3-P5 expect near perfect chromosome arms
< 1GB:	HGAP/PacBio2CA @ 100x PB C3-P5 high quality assembly: contig N50 over 1Mbp
> 1GB:	hybrid/gap filling expect contig N50 to be 100kbp – 1Mbp
> 5GB:	Email mschatz@cshl.edu

Error correction and assembly complexity of single molecule sequencing reads.
Lee, H*, Gurtowski, J*, Yoo, S, Marcus, S, McCombie, WR, Schatz, MC
http://www.biorxiv.org/content/early/2014/06/18/006395

Acknowledgements

Schatz Lab

Rahul Amin
Tyler Gavin
James Gurtowski
Han Fang
Hayan Lee
Maria Nattestad
Aspyn Palatnick
Srividya Ramakrishnan
Zak Lemmon
Eric Biggers
Ke Jiang
Shoshana Marcus
Giuseppe Narzisi
Rachel Sherman
Greg Vurture
Alejandro Wences

CSHL

Hannon Lab
Gingeras Lab
Jackson Lab
Hicks Lab
Iossifov Lab
Levy Lab
Lippman Lab
Lyon Lab
Martienssen Lab
McCombie Lab
Tuveson Lab
Ware Lab
Wigler Lab

IT & Meetings Depts.
Pacific Biosciences
Oxford Nanopore

NSF

National Human
Genome Research
Institute

U.S. DEPARTMENT OF
ENERGY

SFARI
SIMONS FOUNDATION
AUTISM RESEARCH INITIATIVE

Thank you

http://schatzlab.cshl.edu

@mike_schatz