Current Advances in Sequencing Technology

James Gurtowski

Schatz Lab

Outline

1. Assembly Review

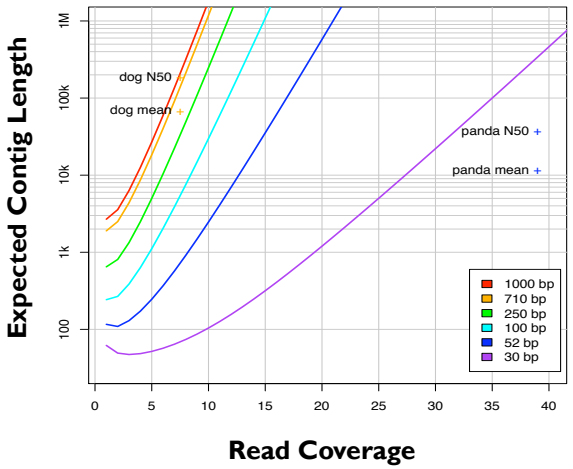
2. Pacbio
   - Technology Overview
   - Data Characteristics
   - Algorithms
   - Results – Assemblies

3. Oxford Nanopore
   - Technology Overview
   - Data Characteristics
   - Algorithms
   - Results – Assemblies

4. Summary

Ingredients for a good assembly

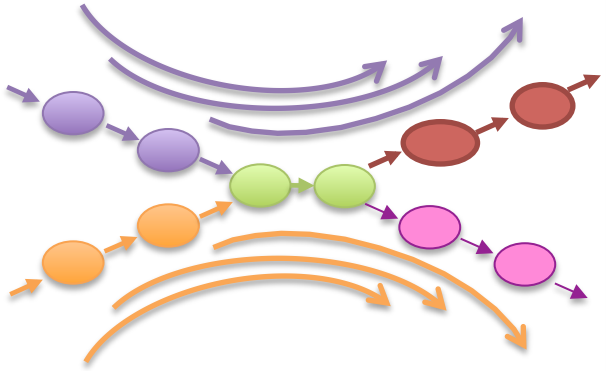
Coverage



High coverage is required

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

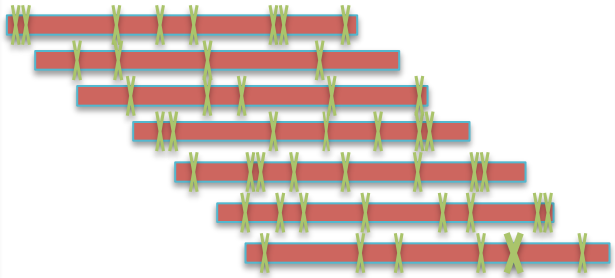
Read Length



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

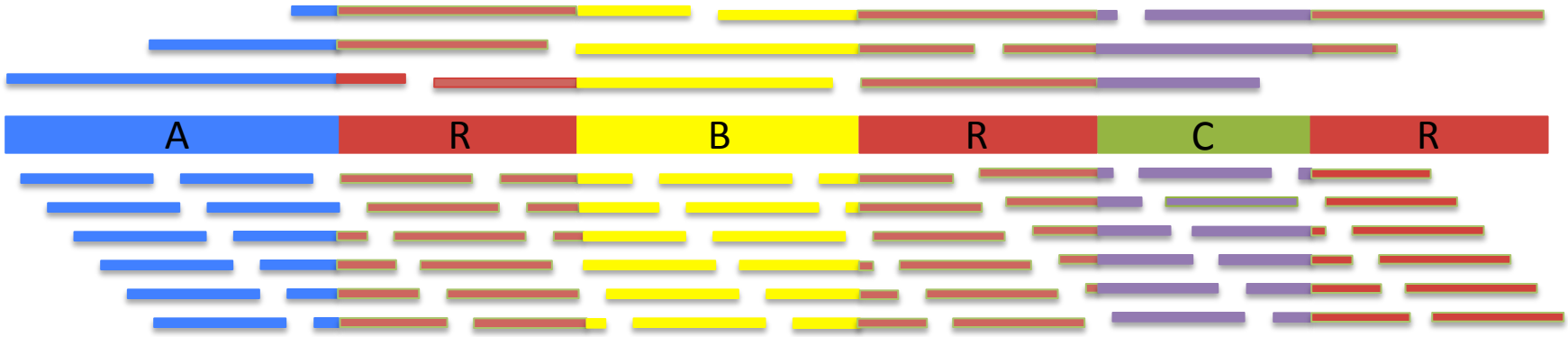


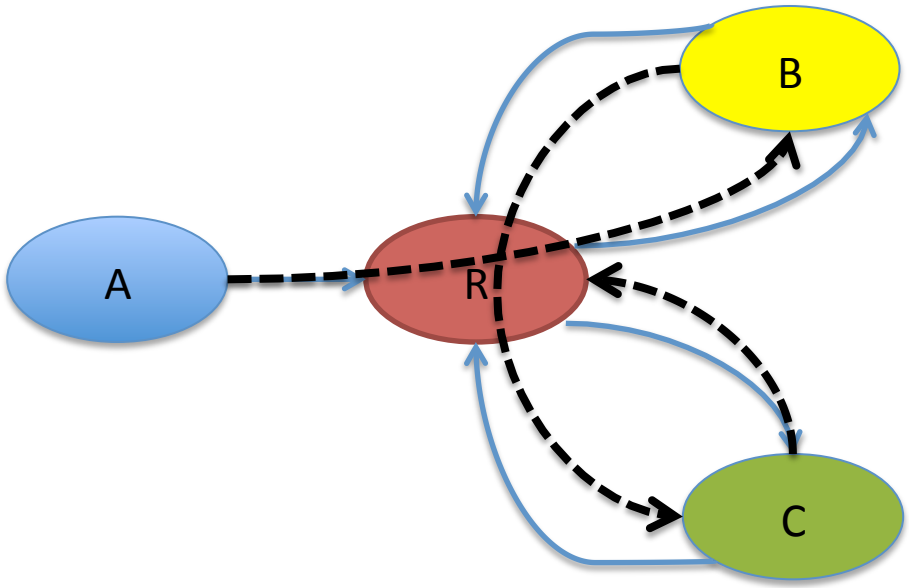
Errors obscure overlaps

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

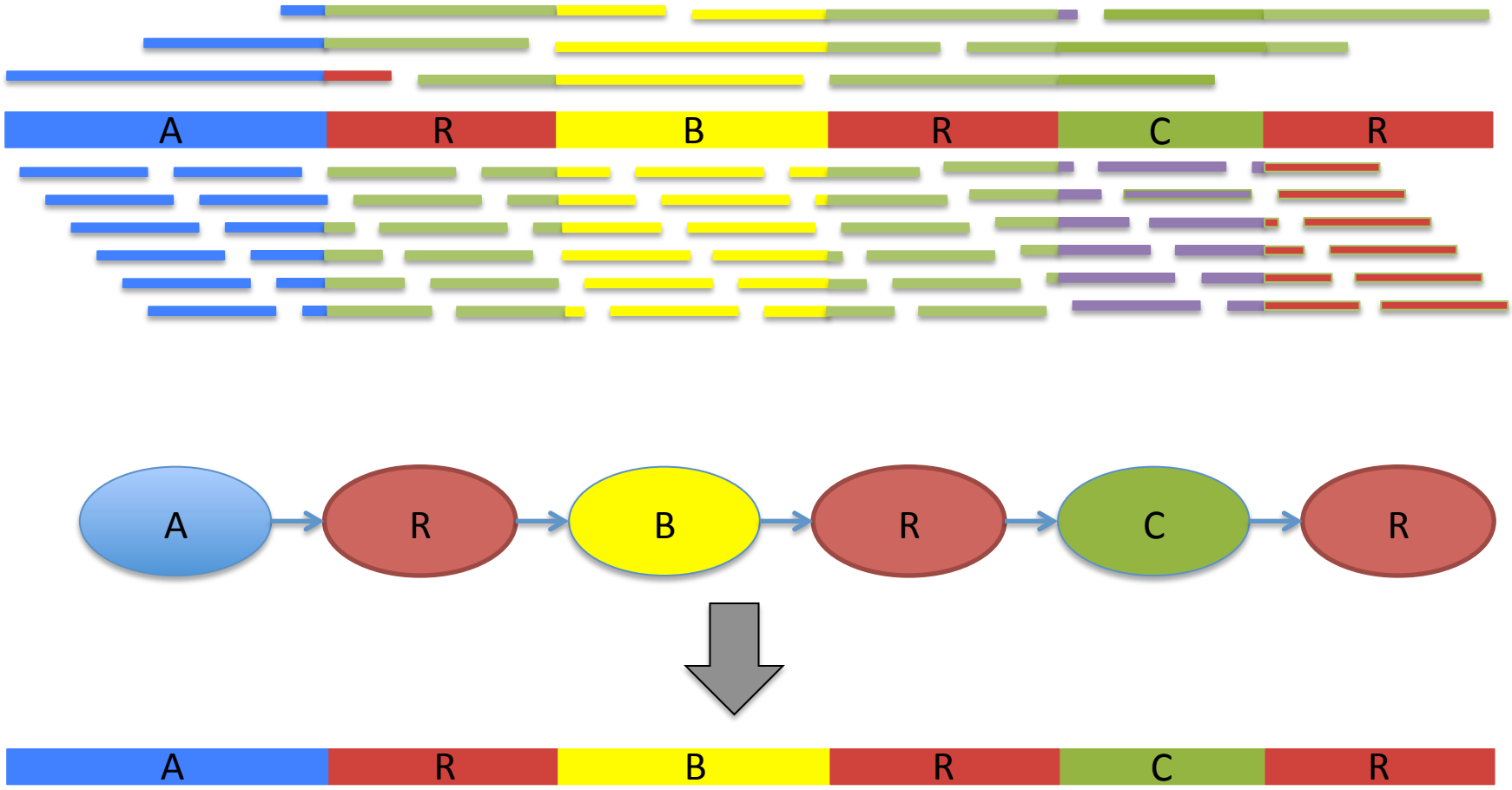
Current challenges in *de novo* plant genome sequencing and assembly
Schatz MC, Witkowski, McCombie, WR (2012) *Genome Biology*. 12:243

Assembly Complexity





Assembly Complexity

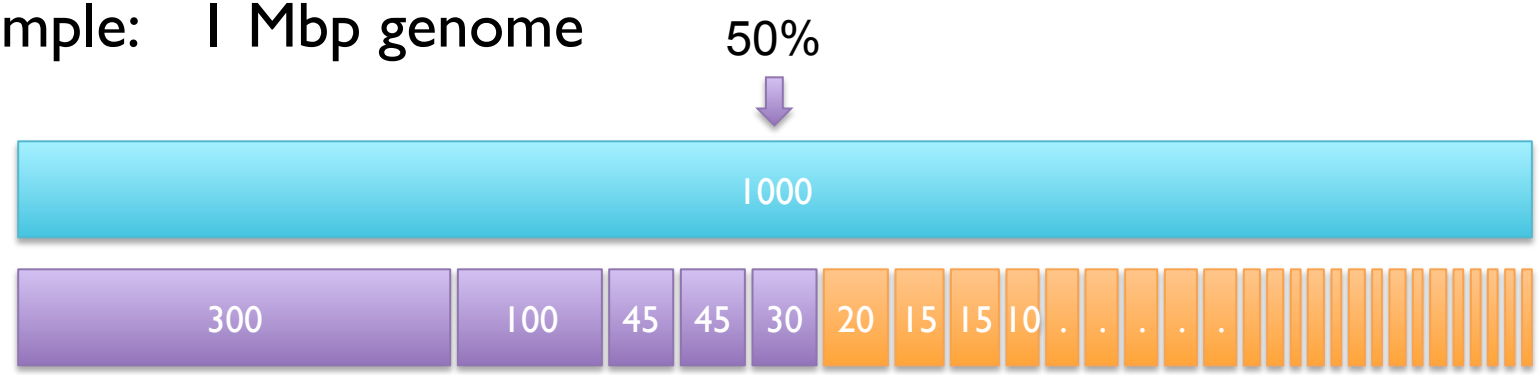


The advantages of SMRT sequencing
Roberts, RJ, Carneiro, MO, Schatz, MC (2013) *Genome Biology.* 14:405

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

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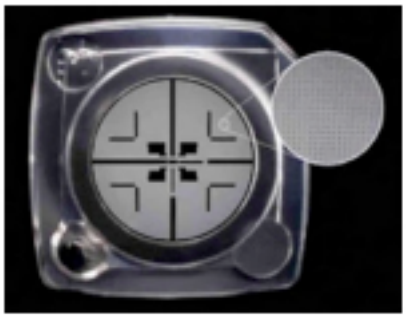
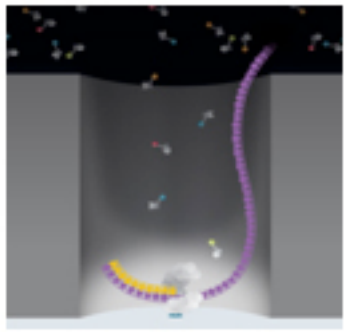
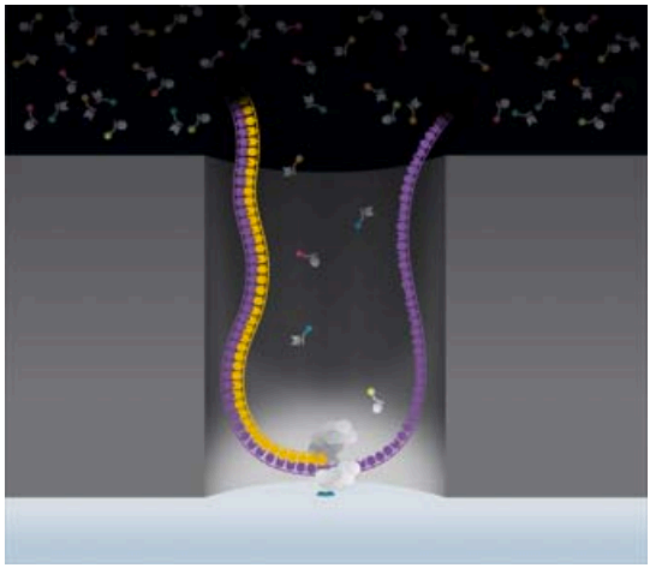
PACIFIC BIOSCIENCES™

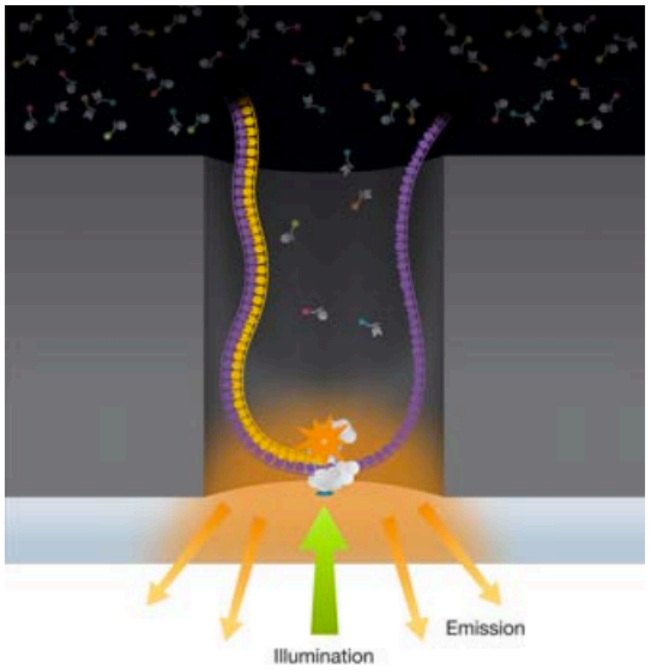


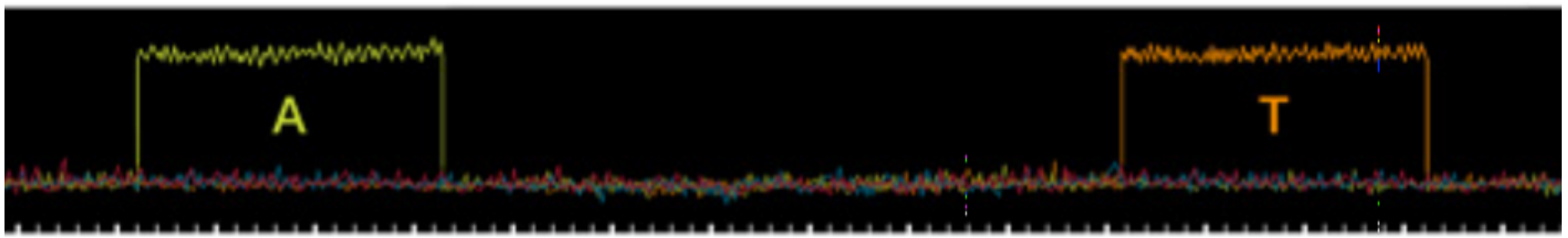


SMRT Sequencing

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

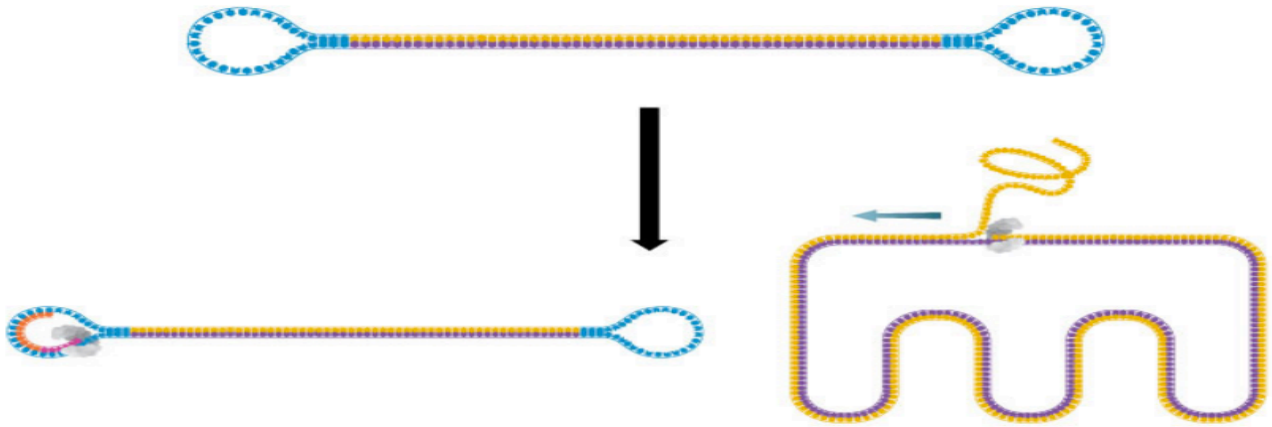






http://www.pacificbiosciences.com/assets/files/pacbio_technology_backgrounder.pdf

SMRT Read Types

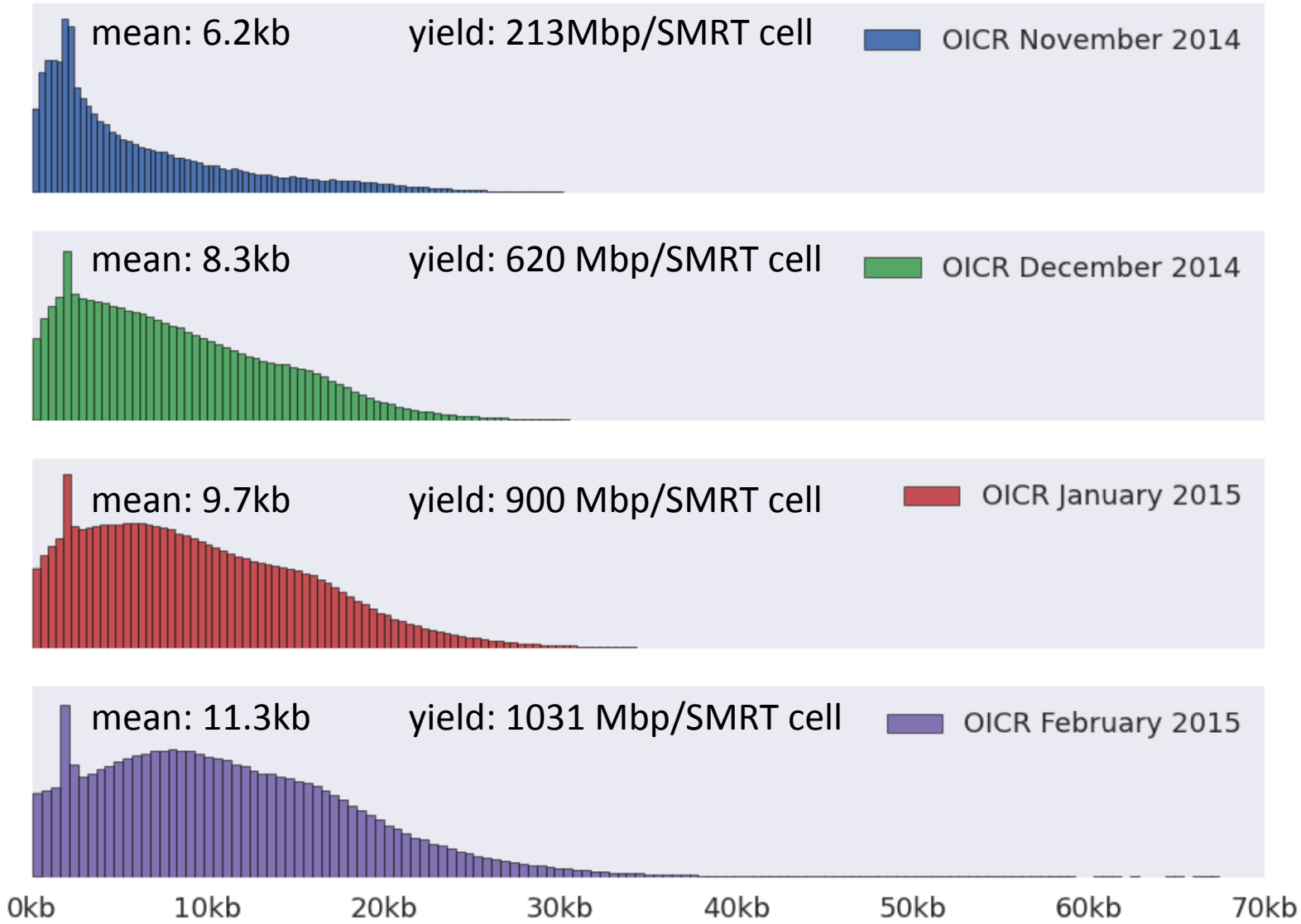


- ***Standard sequencing***
  - Long inserts so that the polymerase can synthesize along a single strand

- ***Circular consensus sequencing***
  - Short inserts, so polymerase can continue around the entire SMRTbell multiple times and generate multiple sub-reads from the same single molecule.
  - Barbell sequence: ATCTCTCTCttttcctcctcctccgttgttgttgttGAGAGAGAT

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

Dramatic changes just by experimenting with library preparation



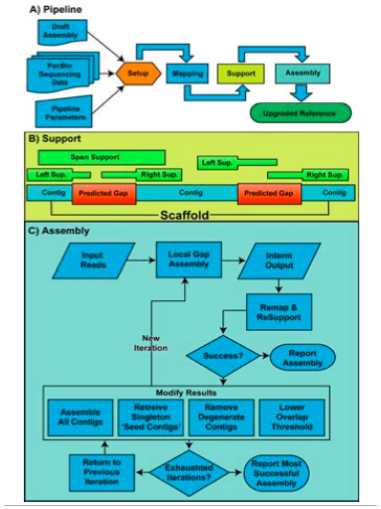
Average Error Rate	16%
Mismatches	30.0%
Deletions	11.5%
Insertions	58.5%

```
> chr7
Length=1090940

 Score =  230 bits (1072),  Expect = 3e-60
 Identities = 612/809 (76%), Gaps = 172/809 (21%)
 Strand=Plus/Minus

Query  28       TTCTCCTTGTTTCTTTTCAACCTATAAAACGTTTTTATTGACGCATAGACAACTAAGCCA  87
                ||||  ||||||| |||||||  |||||   |  |||||||| | |||||||||||| |||
Sbjct  1058792  TTCTTCTTGTTT-TTTTCAA--TATAA---C--TTTATTGA-G-ATAGACAACTAA-CCA  1058744

Query  88       ACAACGAAAACAACCCGGTCAAAGATCGGCGCGTTCTCTGCTACTACCATTCTTCGCTATG  147
                | || ||||  ||  |||||||||||    |||| ||| |||||| ||  | ||||||
Sbjct  1058743  A-AAAGAAA--AA--CGGTCAAAGAT----GCGTTCT-CTACTA-CA--C-TCGCTA--  1058700

Query  148      CTGCAGGCTACTTGCGTAGCTTATTTTTCAGCGAGCTTCTACCGTCAAGGTTTCAGCTAT  207
                ||||| ||||| |||   || ||||||||| | |||  || ||  ||| ||||||||||
Sbjct  1058699  CTGCA-GCTAC-TGC---GC-TATTTTTCA-C-AGC--CT-CC--CAA-GTTTCAGCTAT  1058654

Query  208      TGGTGAACTAGCCTTTAAGTCTGGGTGTGCTAAGTGAACAACGATAGGTAGCTGTGTAAG  267
                |||||||||||||||| | ||||||| | ||  |||||||||| |||| || ||||||
Sbjct  1058653  TGGTGAACTAGCCTTTAACT-TGGGTGT-C-AA--GAACAACGAT-GGTA-CT-TGTAAG  1058602

Query  268      TCCACTCCTTCCCGAACTTATTGTAACGAAATACAAGGCTTTGACTATAGCAGCTTCCAA  327
                ||||   |||| || |||||   | |||| ||||| |||||||  |      ||||| |
Sbjct  1058601  TCCA---CTTC-CG-AC-TATGAAACCGAATTACAA-GCTTTGAAGAGCTACACTTCC-A  1058550

Query  328      CCGGTCTATAAAGTTATACTGCTGTGCCTCAGATCTGTAACAGCTTTTGCAAAACTTAGG  387
                || |||   |||||| ||| ||  |||||||||| ||||||| | |||||||||||| |
Sbjct  1058549  CC-GTC---AAAGTT-TAC-GC--TGCCTCAGAT-TGTAACA-C-TTTGCAAAACTTA-G  1058502

Query  388      GTCCTGCGCCTGTCTTCCTGAAGGGCTGAGGACTATTTACTCATGCTTATAGTCGGTGTTT  447
                |||||       |||||||  |||||||   ||||||| || ||| | |||||||||||
Sbjct  1058501  GTCCTG-------CTCCTGAA--GCTGAGG--GATTTACT-AT-CTT-T-GTCGGTGTTT  1058457

Query  448      GGCGGCAACAGACCGACAGTGCAGTGATACGCTGCTG-AAAGGCTGCTTCGTTTGCAAAA  506
                |||  ||||||| ||||||| || |  |||||||||| ||||||||    |||||||||
Sbjct  1058456  GGC--CAACAGA-CGACAGT-CA-T--TACGCTGCTGAAAAGGCTGC----TTTGC-AAA  1058409

Query  507      CCTAATTTGGCGCTAAAAATTAAAAGCAATCCACTGTTAGGCTGGTTTTCTTGGTTGGGA  566
                ||| ||||   || ||||||| |||| ||||||||||  ||||| ||||||||||||
Sbjct  1058408  CCT-ATTT---GCCAAAAATT-AAAG-AATCCACTGTT--GCTGG-TTTCTTGGTTGG--  1058360
```

Error Profile Dominated by **Insertions**

Long Read Correction Algorithms

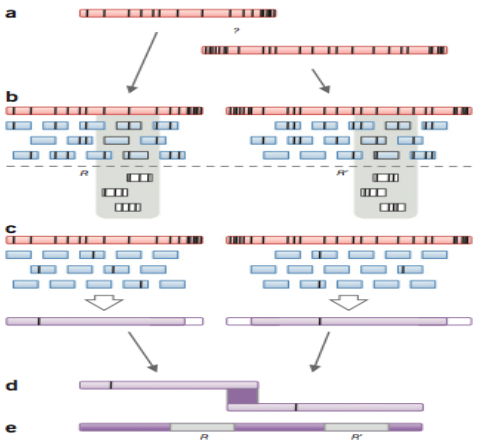
PBJelly



Gap Filling and Assembly Upgrade

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

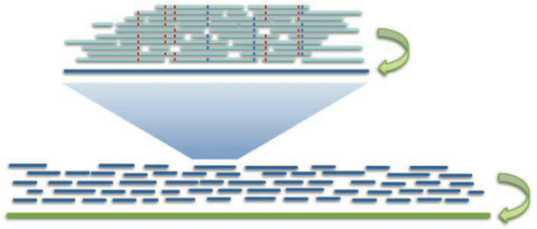
PacBioToCA & ECTools



Hybrid Error Correction

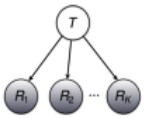
Koren, Schatz, *et al* (2012)
Nature Biotechnology. 30:693–700

HGAP & Quiver



$$\Pr(\mathbf{R} \mid T)$$

$$\Pr(\mathbf{R} \mid T) = \prod_k \Pr(R_k \mid T)$$



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

LR-only Correction & Polishing

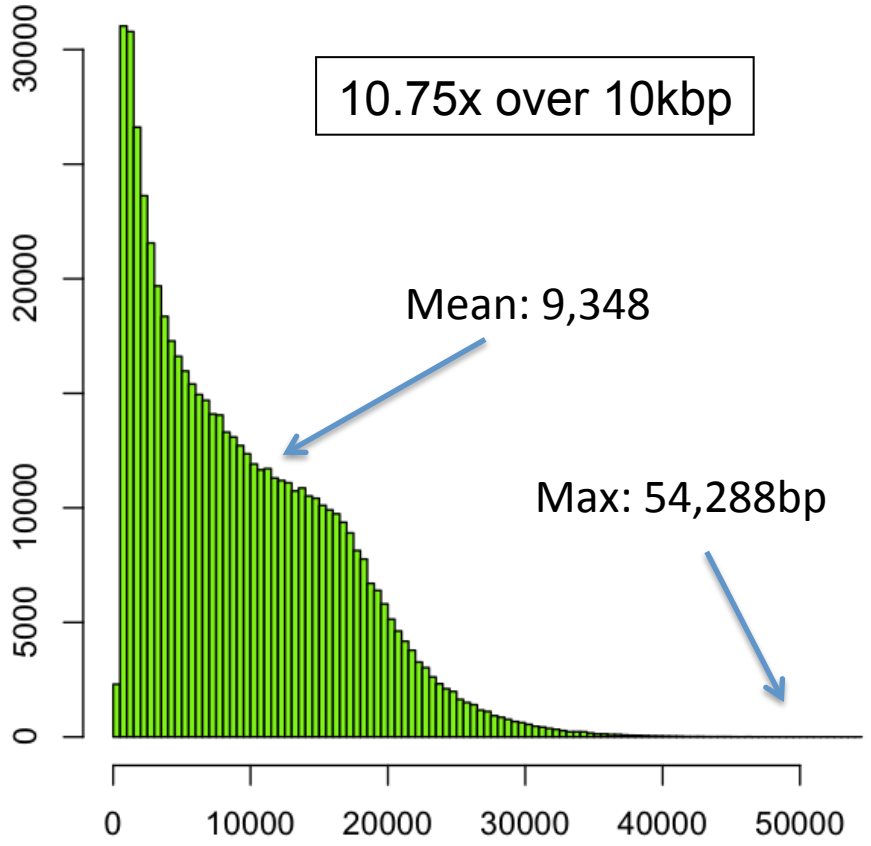
Chin *et al* (2013)
Nature Methods. 10:563–569

< 5x Long Read Coverage > 50x

O. sativa pv Indica (IR64)

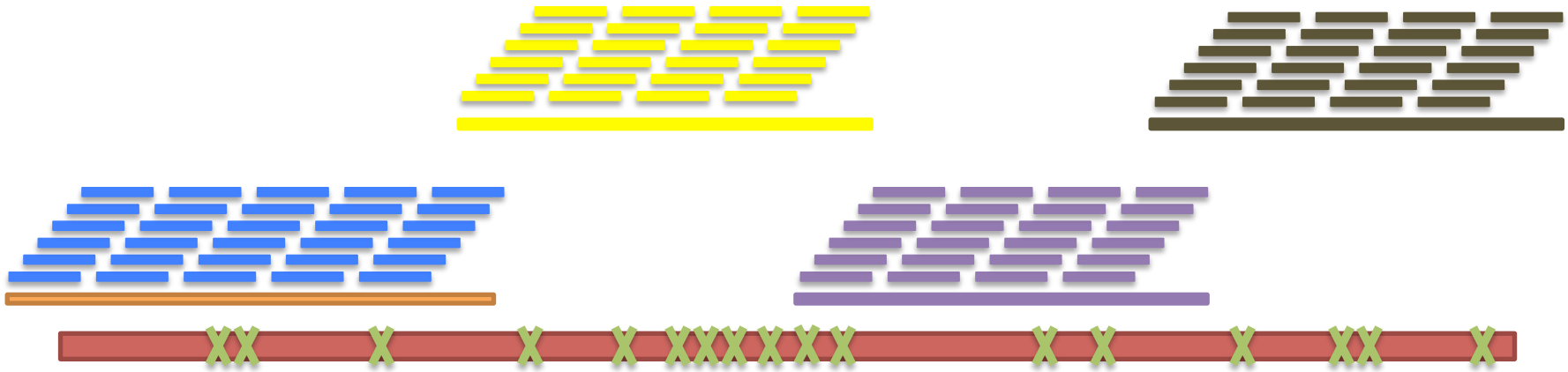
Genome size: ~370 Mb
Chromosome N50: ~29.7 Mbp

Assembly	Contig NG50
"ALLPATHS-recipe" 50x 2x100bp @ 180 36x 2x50bp @ 2100 51x 2x50bp @ 4800	18,450
MiSeq Fragments 25x 456bp (3 runs 2x300 @ 450 FLASH)	19,078
PacbioToCA – 47 SMRTCells 10.7x @ 10kbp	144,042
ECTools - 47 SMRTCells 10.7x @ 10kbp	????



ECTools: Error Correction with pre-assembled reads

https://github.com/jgurtowski/ectools



Short Reads -> Assemble Unitigs -> Align & Select - > Error Correct

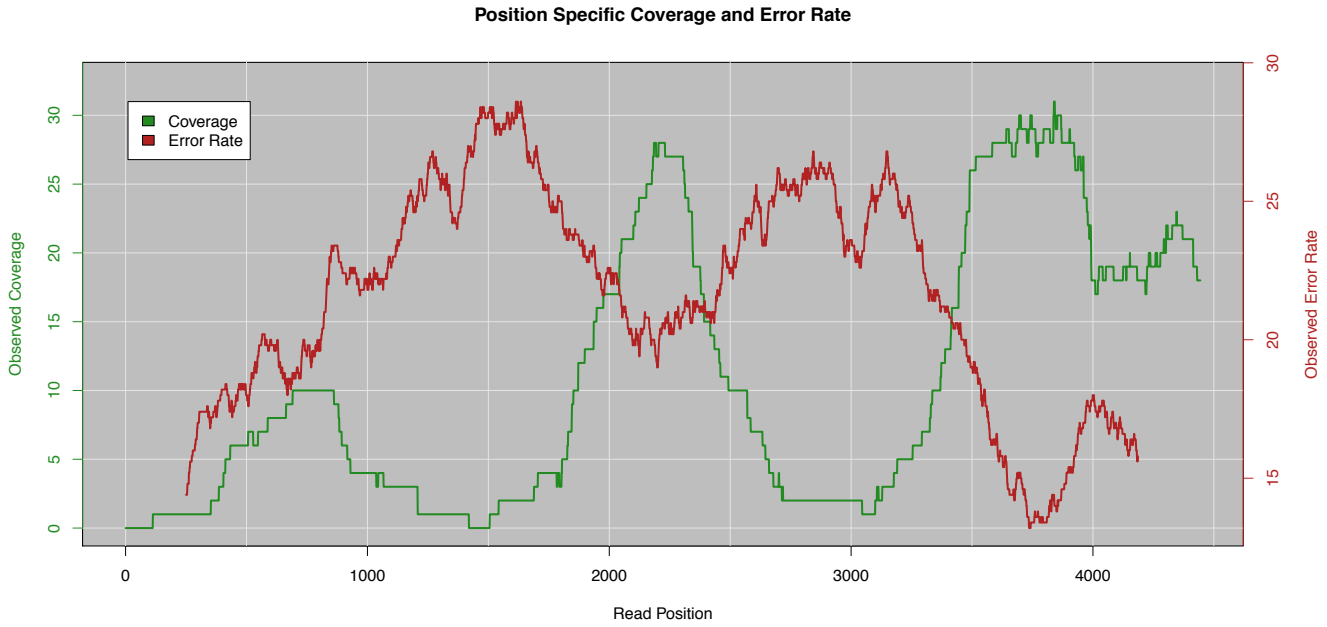
Can Help us overcome:

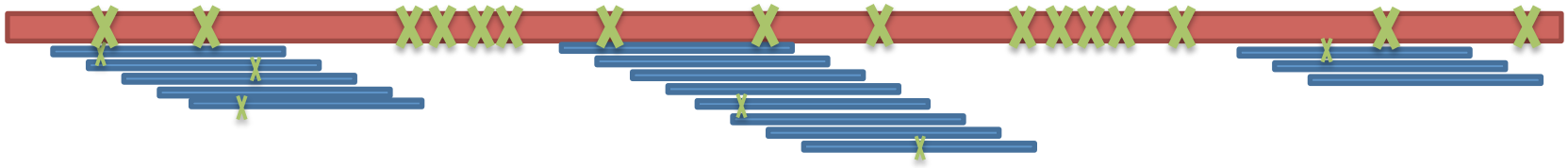
1. Error Dense Regions – Longer sequences have more seeds to match
2. Simple Repeats – Longer sequences easier to resolve

However, cannot overcome Illumina coverage gaps & other biases

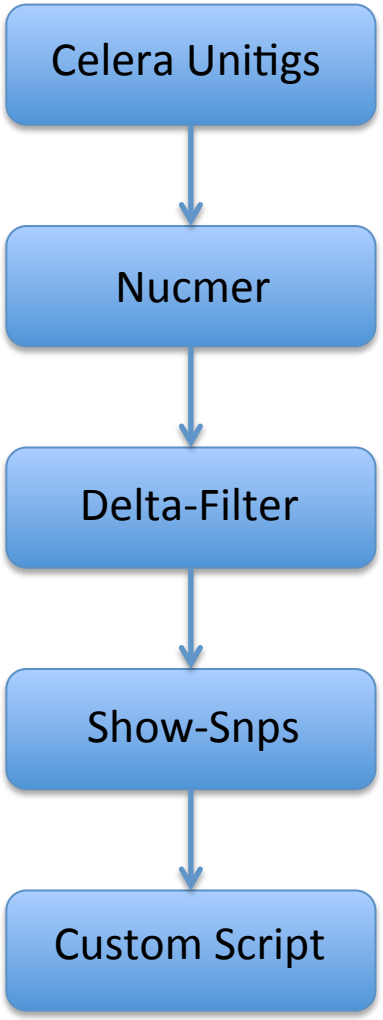
Low Coverage Regions

1. Simple Repeats – Kmer Frequency Too High to Seed Overlaps
2. GC Rich Regions – Known Illumina Bias
3. **Error Dense Regions – Difficult to compute overlaps with many errors**





ECTools Pipeline



Generate Unitigs with Celera Using Illumina or another high identity sequencing technology

Align Unitigs to Pacbio Reads With Nucmer

Use Delta-Filter to Generate Unitig Layout

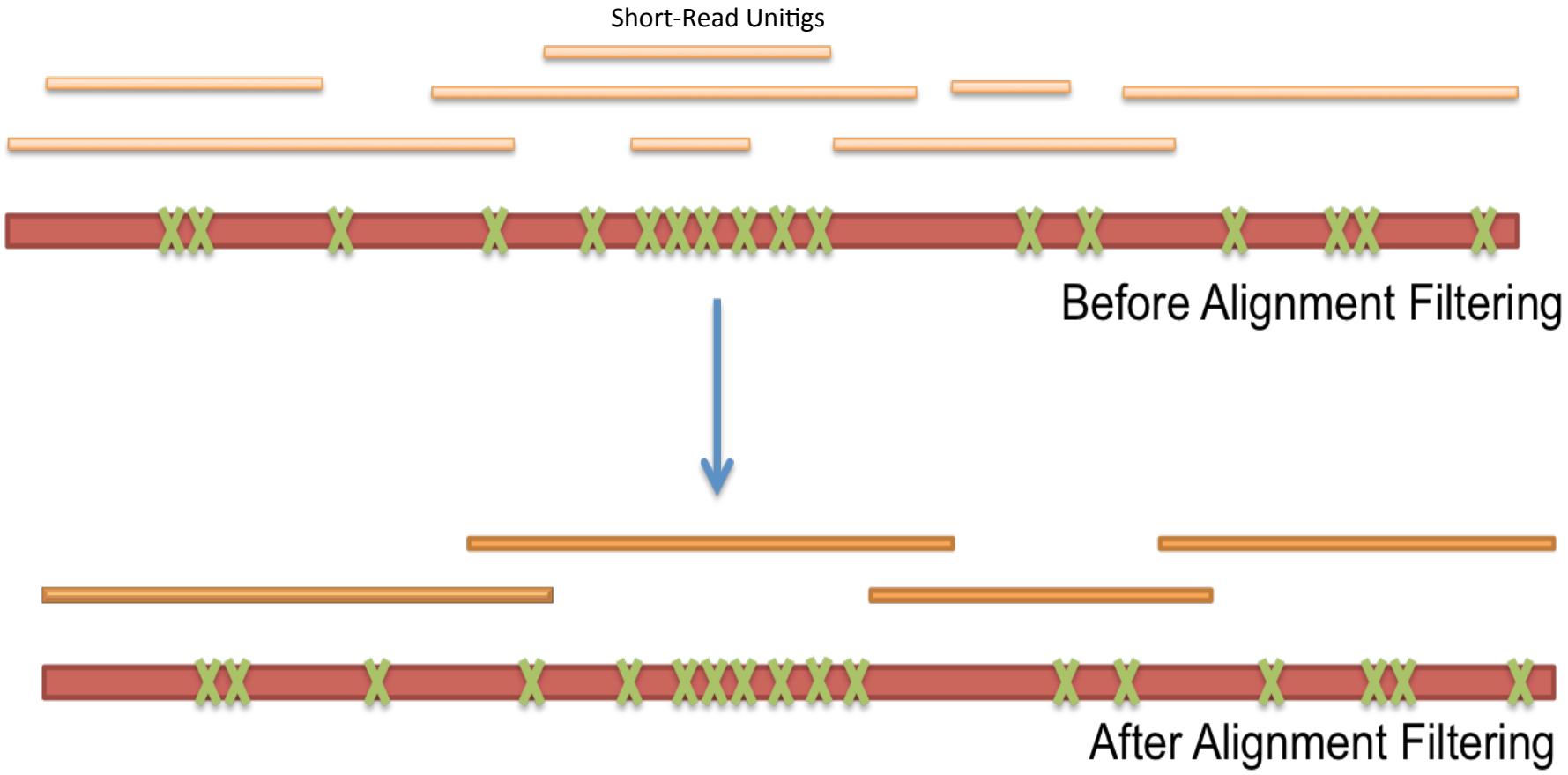
Show-Snps shows differences between trusted Illumina Unitig Sequence and Pacbio Read

Script to “Correct” Pacbio Read

Note: Reads are never split or trimmed

Delta-Filter Alignment filtering

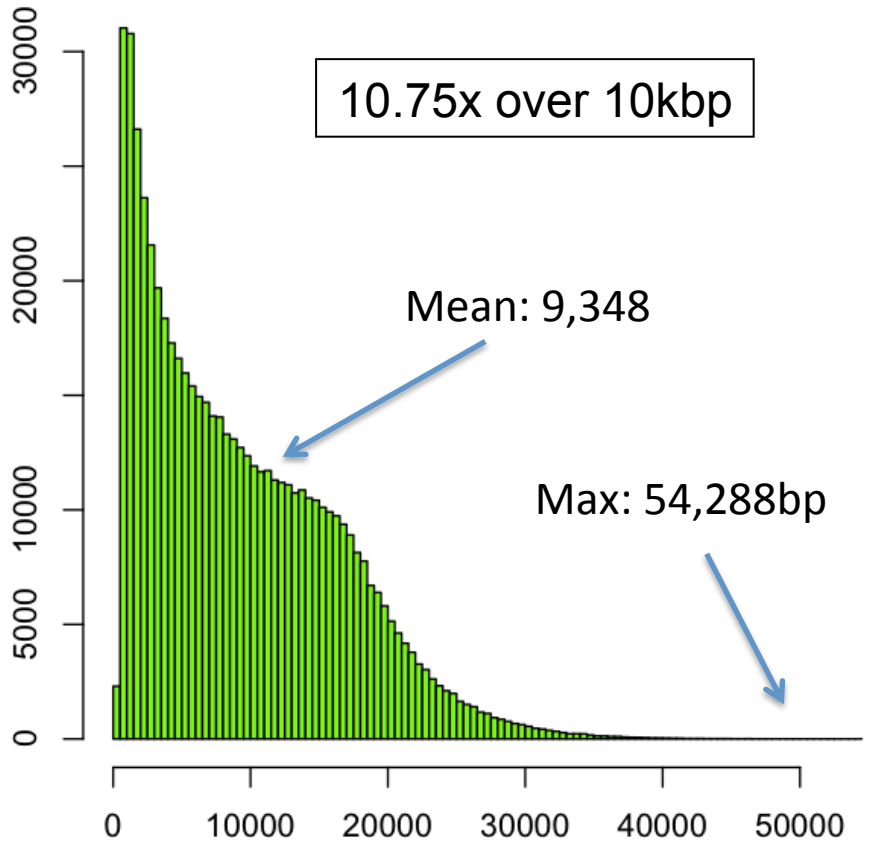
Uses Dynamic Programming (Longest Increasing Subset) to find the longest mutually consistent subset of unitigs with respect to the Pacbio Read



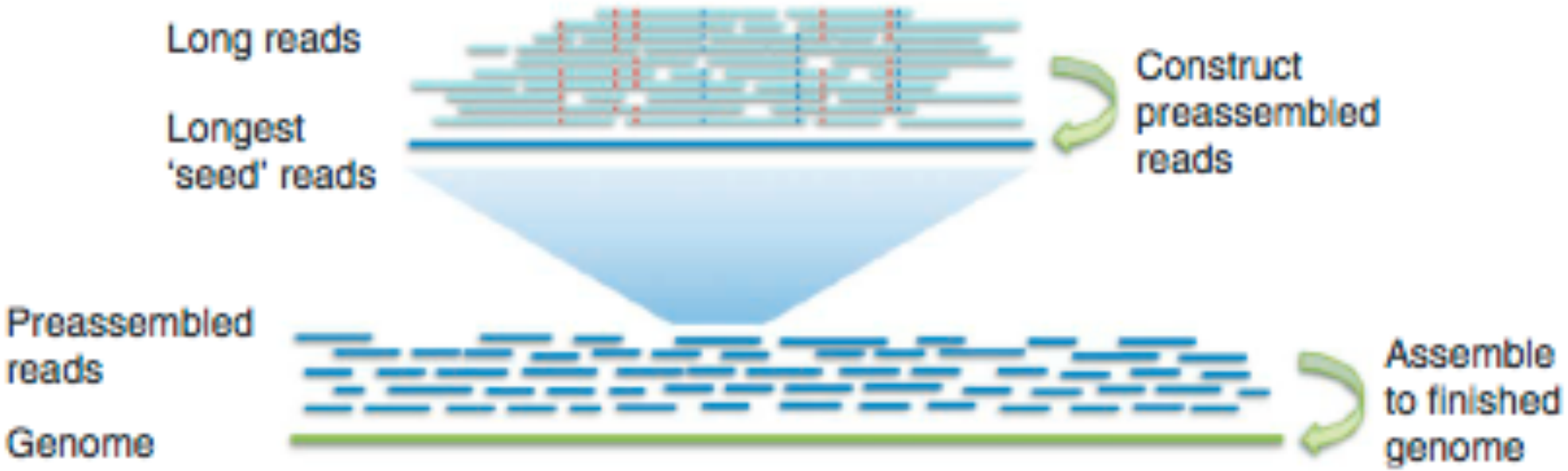
O. Sativa Indica (IR64)

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

Assembly	Contig NG50
"ALLPATHS-recipe" 50x 2x100bp @ 180 36x 2x50bp @ 2100 51x 2x50bp @ 4800	18,450
MiSeq Fragments 25x 456bp (3 runs 2x300 @ 450 FLASH)	19,078
PacbioToCA – 47 SMRTCells 10.7x @ 10kbp	144,042
ECTools - 47 SMRTCells 10.7x @ 10kbp	272,137



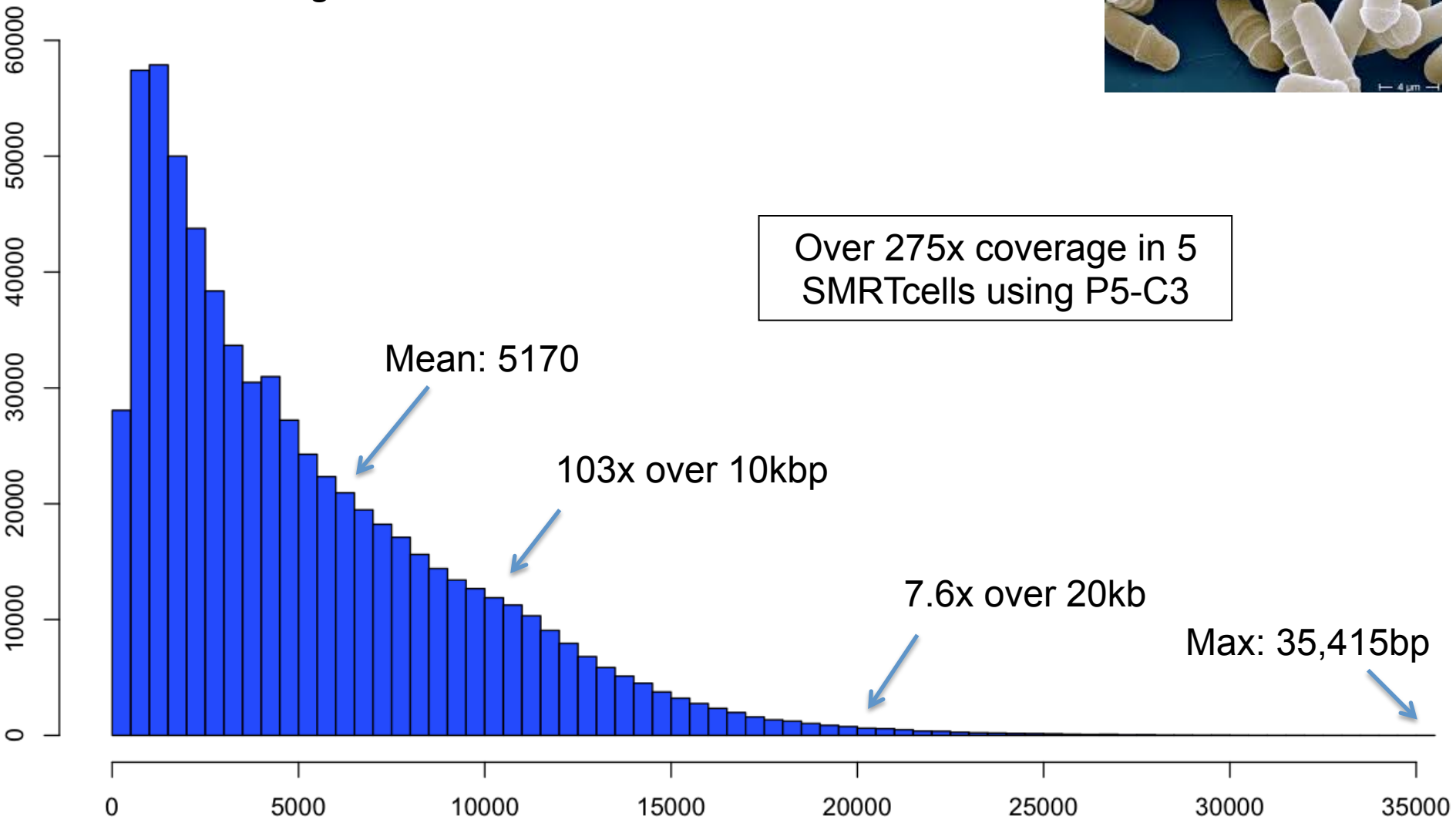
HGAP Error Correction



S. pombe dg21

PacBio RS II *sequencing* at CSHL

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



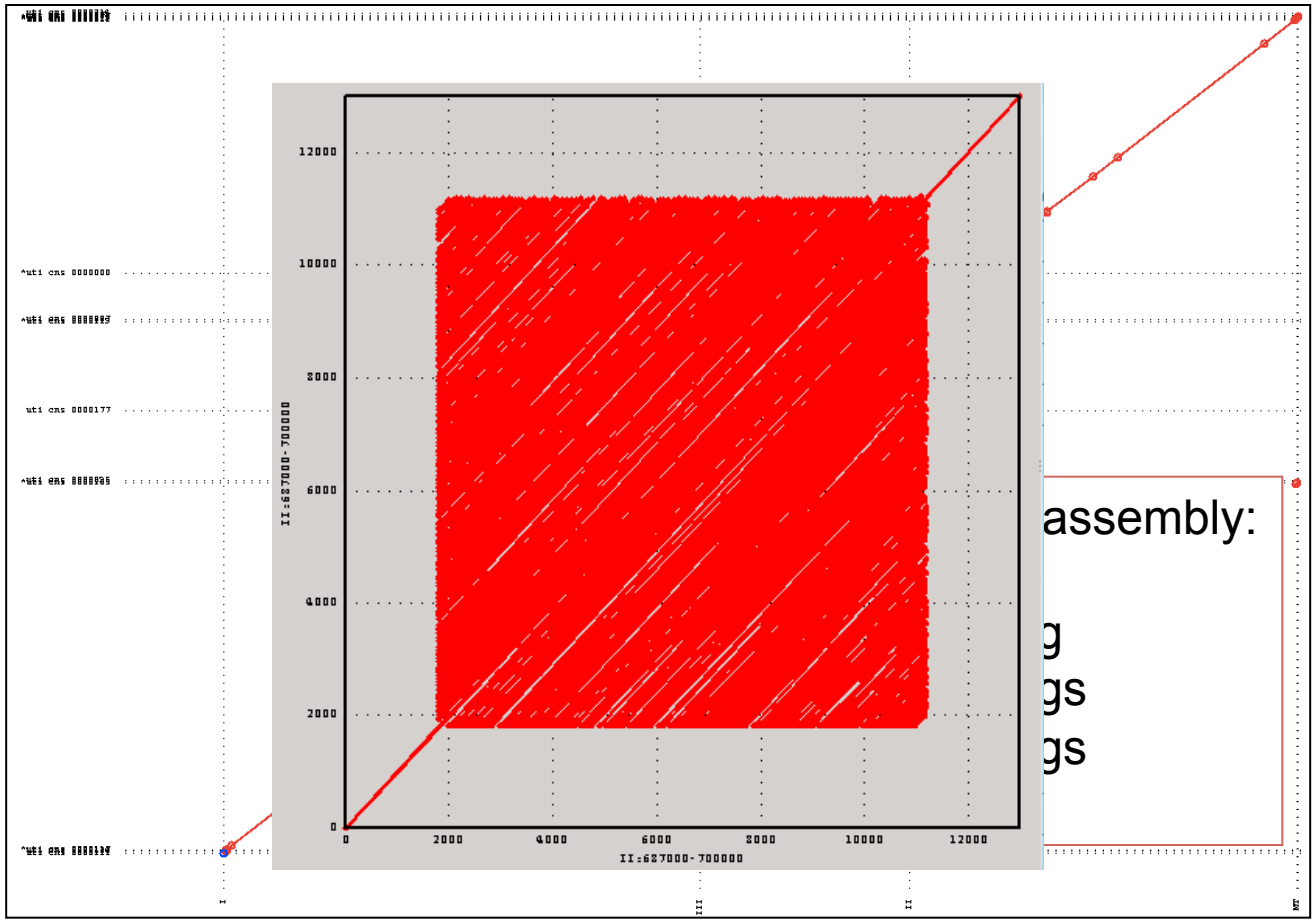
S. pombe dg21

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



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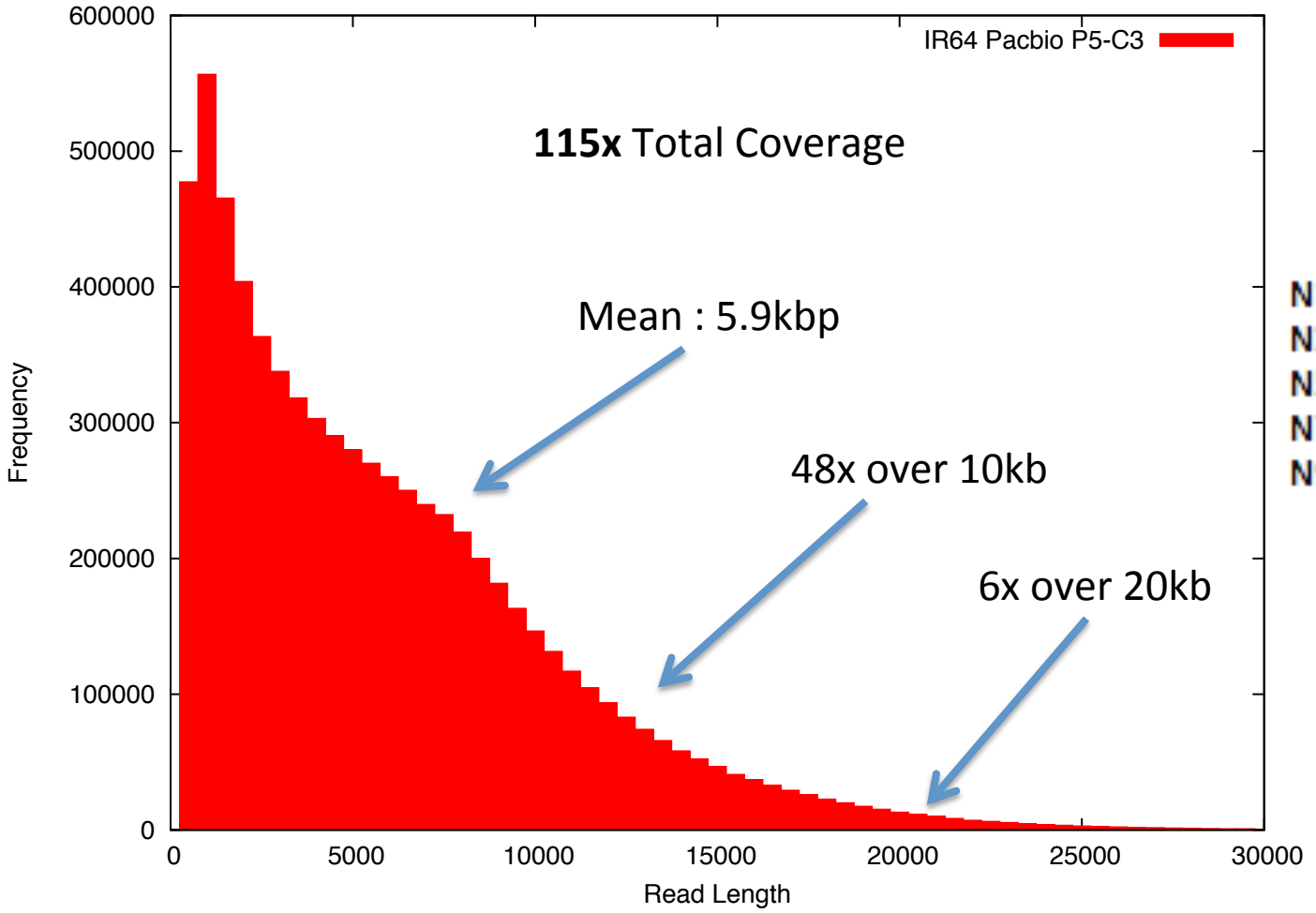


Assembly Results

```
N10=10425102 N10cnt=3
N25=6571607 N25cnt=11
N50=3900937 N50cnt=29
N75=1783229 N75cnt=66
N90=859087 N90cnt=108
```

Dazcon

Eugene W Myers Jr
thegenemyers

github SOCIAL CODING

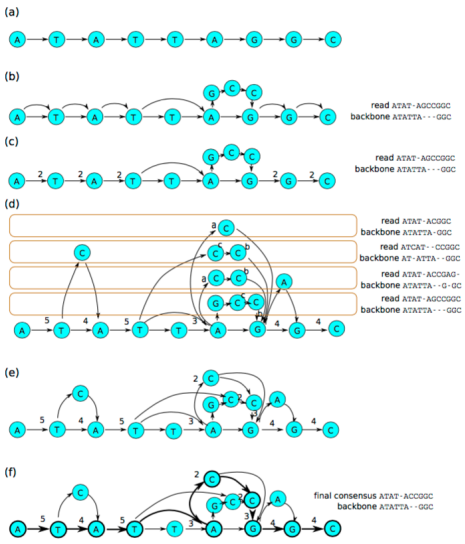
PACIFIC BIOSCIENCES™

thegenemyers / **DALIGNER**

PacificBiosciences / **pbdagcon**

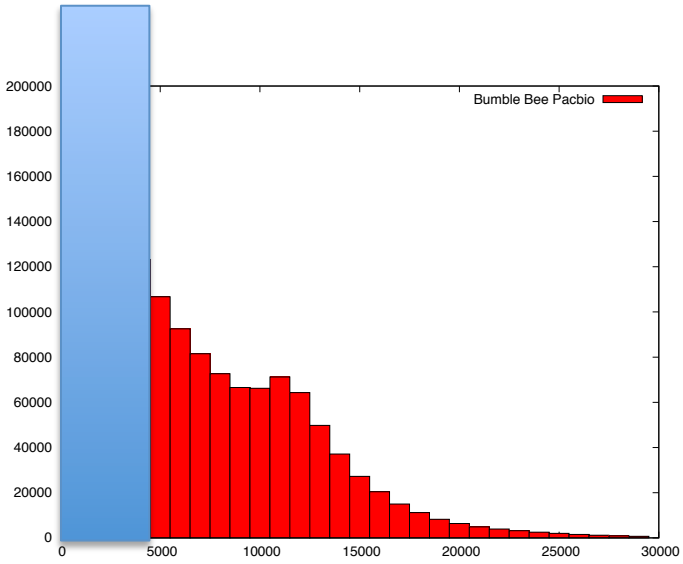
```
                  12645
A       ---------+====>
B  <====+---------
     9017
```

```
dazcon -ox -j 4 -s subreads.db -a subreads.las > corrected.fasta
```



Bumble Bee Genome Assembly

Discard reads <5kb



illumina ✚

```
n=369356 [26, 453616] 767.79 +/- 5960.50 sum=283588978 cov=1.13

N10=124463 N10cnt=145
N25=72986 N25cnt=547
N50=35935 N50cnt=1792
N75=13011 N75cnt=4670
N90=3553 N90cnt=9793
```

PACBIO® ✚ **Dazcon**

```
n=26131 [7976, 2171155] 21683.57 +/- 67351.74 sum=566613279 cov=2.27

N10=1219658 N10cnt=17
N25=669835 N25cnt=60
N50=359646 N50cnt=191
N75=183176 N75cnt=436
N90=81409 N90cnt=725
```

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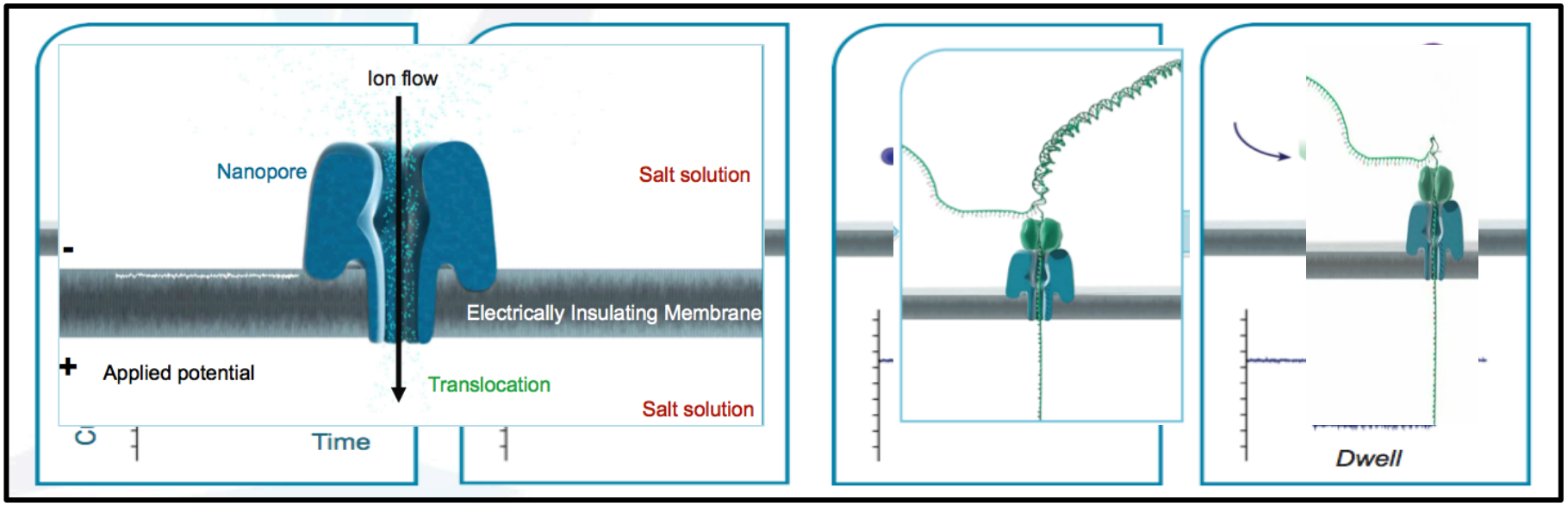
Oxford Nanopore MinION

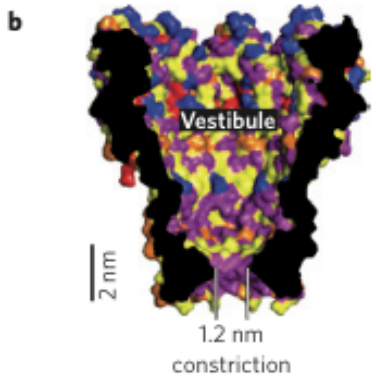
- MAP Program
- Thumb drive sized sequencer powered over USB
- Senses DNA by measuring changes to ion flow
- Reads both DNA Strands (2D)



Advantages And Challenges of Nanopore DNA Sequencing

Advantages

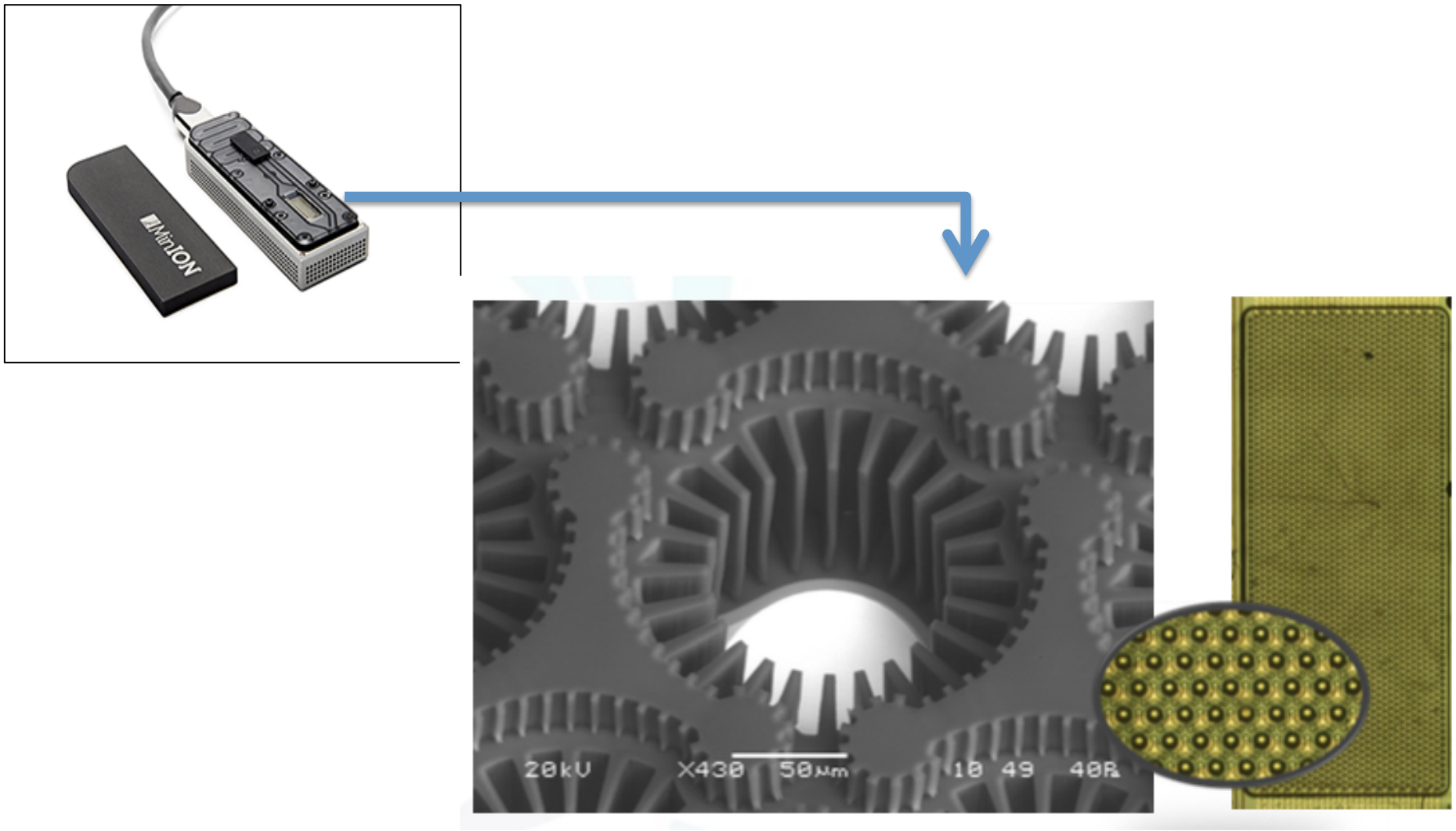
- Label-free
- Amplification-free
- Single-Molecule
- High-Throughput
- Inexpensive Instrument
- Simple/quick Sample Prep
- Produces Long Reads



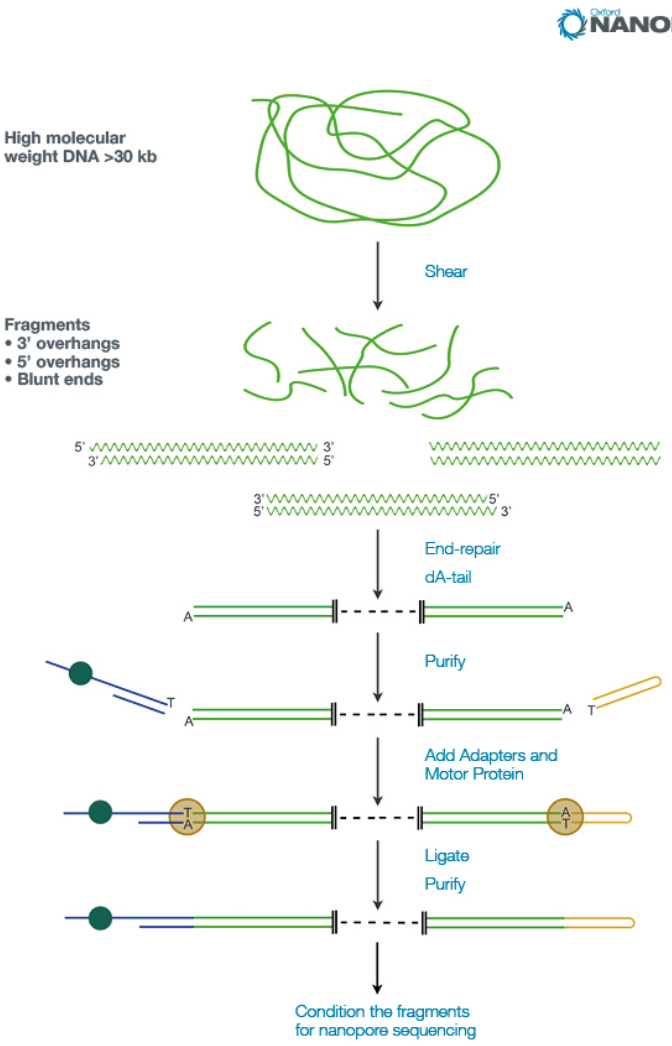
Challenges

- Controlling rate at which DNA translocates through the pore (1base/microsecond too fast to accurately measure current change)
- Pore does not have single base resolution (complicates basecalling and makes it hard to deal with modified bases)
- Commercially: Biological pores are sensitive to pH, temperature, salt concentration

Under the hood of the MinION

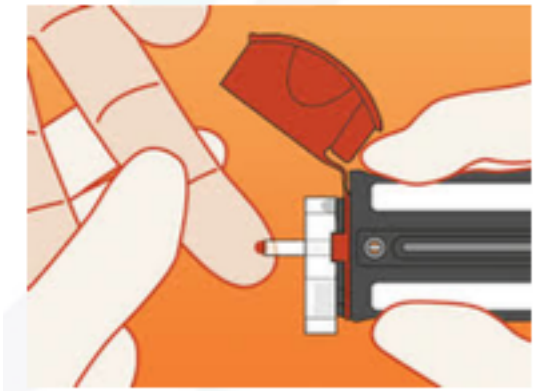


Oxford Nanopore DNA Prep

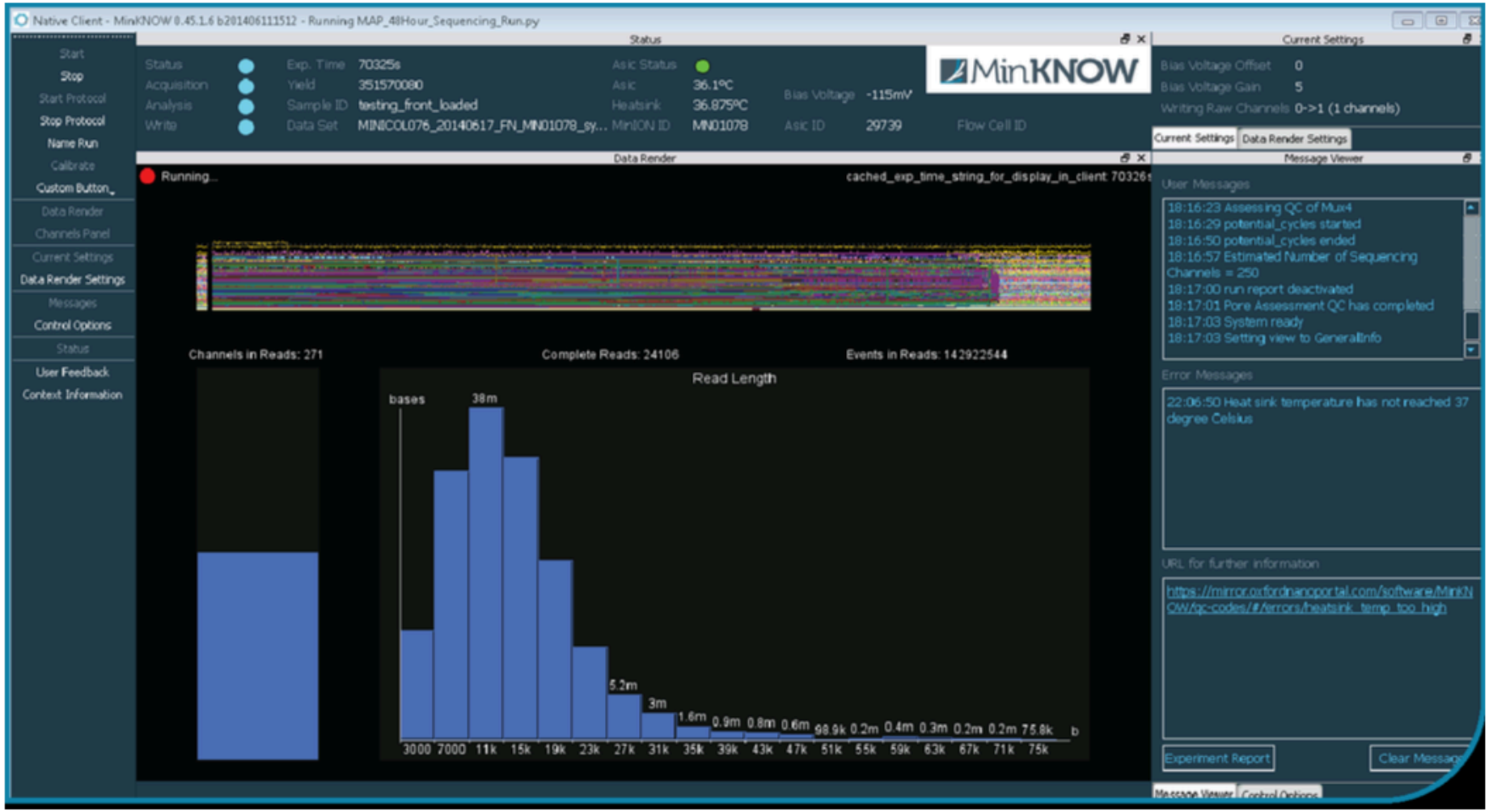


Simple DNA prep

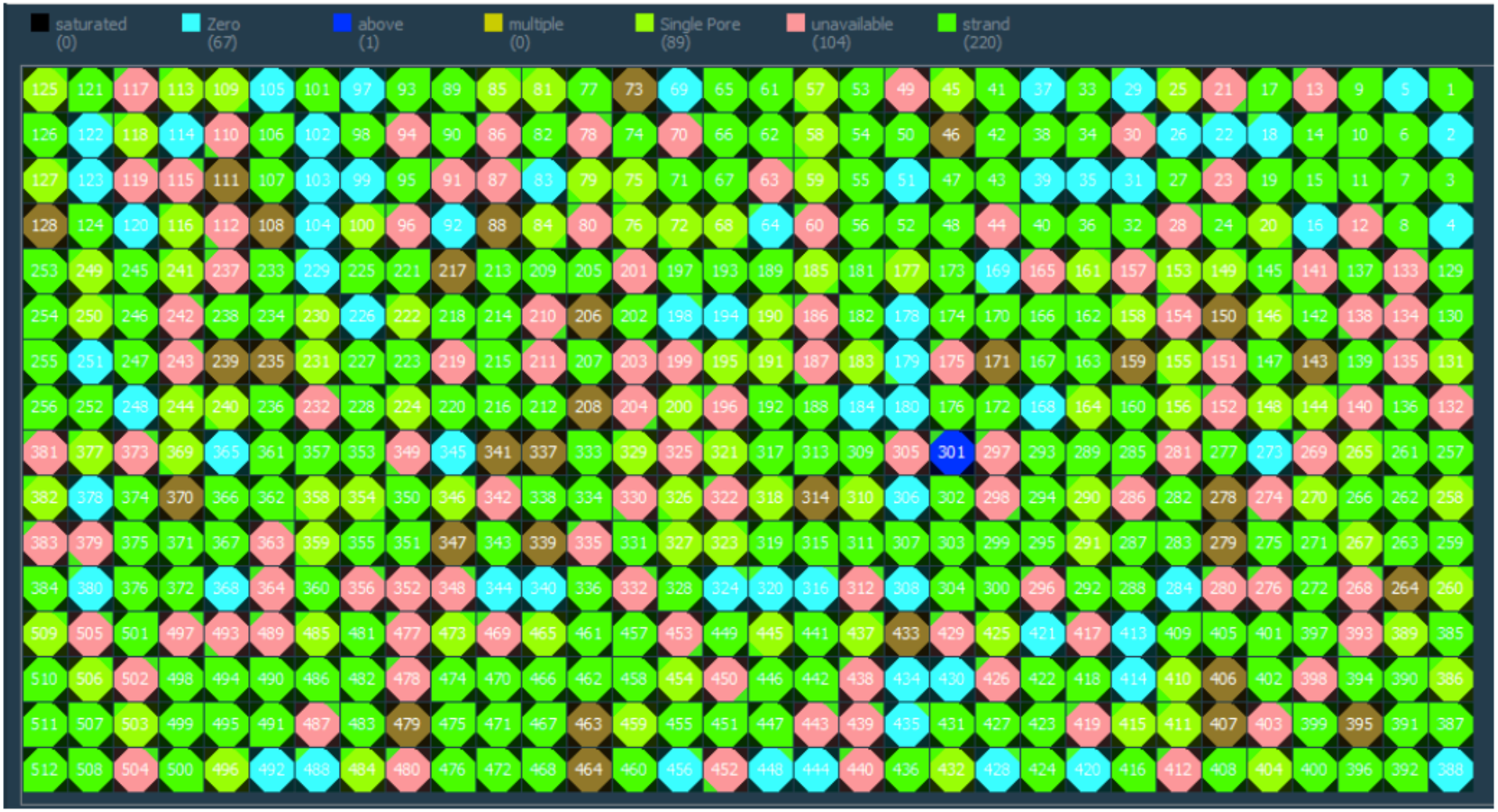
Can do it in the field?



Nanopore Desktop Software



View of Pore Activity

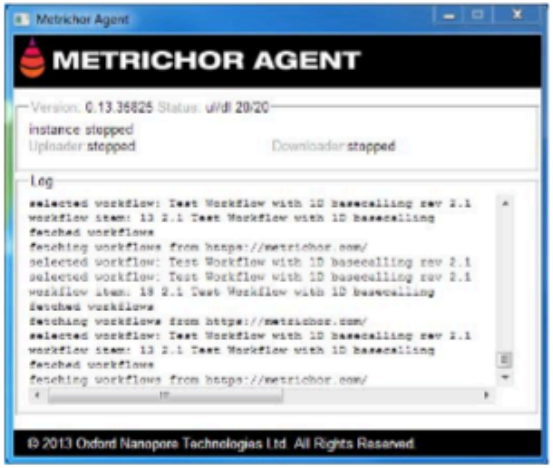


Base Calling

Raw Signal data contained in fast5 (hdf5) files on local machine

Name	Date modified	Type	Size
TEST12345_ch1_file1	14/04/2014 13:38	Fast5 file	217 KB
TEST12345_ch1_file2	14/04/2014 13:38	Fast5 file	210 KB
TEST12345_ch1_file3	14/04/2014 13:38	Fast5 file	276 KB
TEST12345_ch1_file4	14/04/2014 13:39	Fast5 file	242 KB
TEST12345_ch2_file1	14/04/2014 13:39	Fast5 file	204 KB
TEST12345_ch2_file2	14/04/2014 13:39	Fast5 file	243 KB
TEST12345_ch2_file3	14/04/2014 13:39	Fast5 file	246 KB
TEST12345_ch2_file4	14/04/2014 13:39	Fast5 file	191 KB
TEST12345_ch3_file1	14/04/2014 13:39	Fast5 file	369 KB
TEST12345_ch3_file2	14/04/2014 13:40	Fast5 file	309 KB
TEST12345_ch3_file3	14/04/2014 13:40	Fast5 file	365 KB
TEST12345_ch3_file4	14/04/2014 13:40	Fast5 file	313 KB
TEST12345_ch4_file1	14/04/2014 13:40	Fast5 file	464 KB
TEST12345_ch4_file2	14/04/2014 13:40	Fast5 file	245 KB
TEST12345_ch4_file3	14/04/2014 13:40	Fast5 file	239 KB
TEST12345_ch4_file4	14/04/2014 13:41	Fast5 file	249 KB
TEST12345_ch5_file1	14/04/2014 13:41	Fast5 file	248 KB
TEST12345_ch5_file2	14/04/2014 13:41	Fast5 file	275 KB
TEST12345_ch5_file3	14/04/2014 13:41	Fast5 file	276 KB
TEST12345_ch5_file4	14/04/2014 13:41	Fast5 file	183 KB

Local Software Agent facilitates data transaction with cloud basecaller



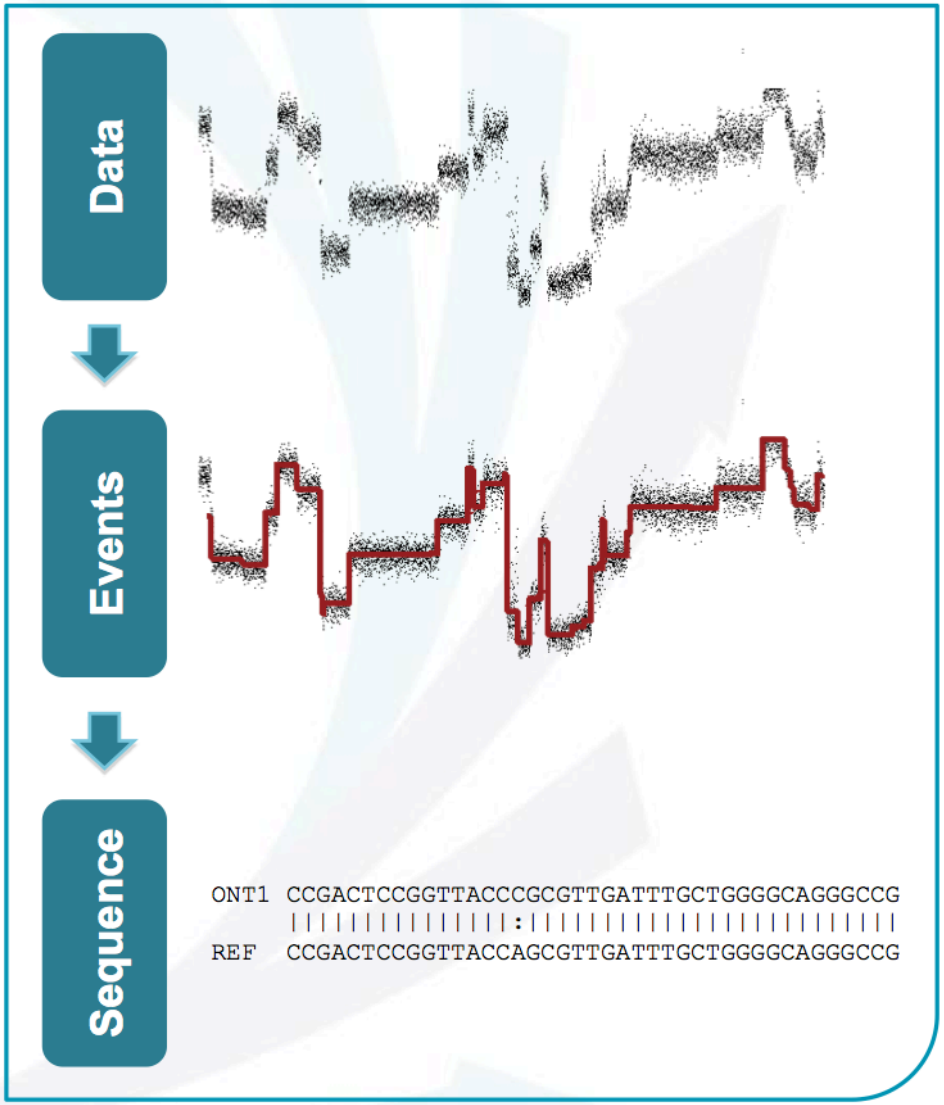
upload reads

Download fast5 with called bases

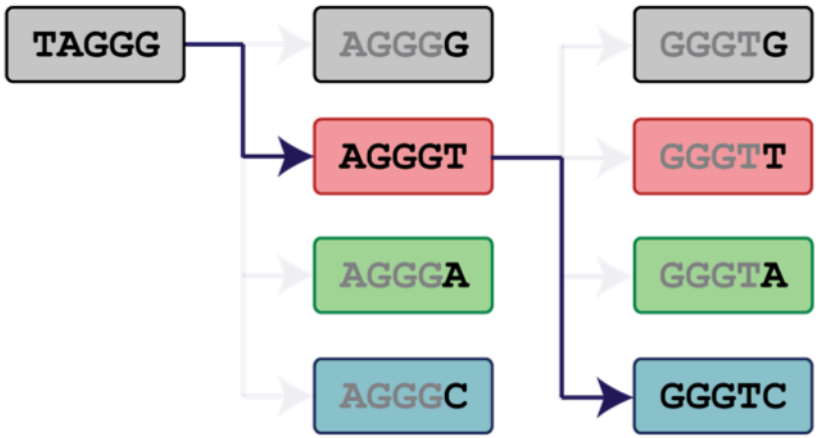
amazon web services

Base Calling in Cloud

Nanopore Basecalling



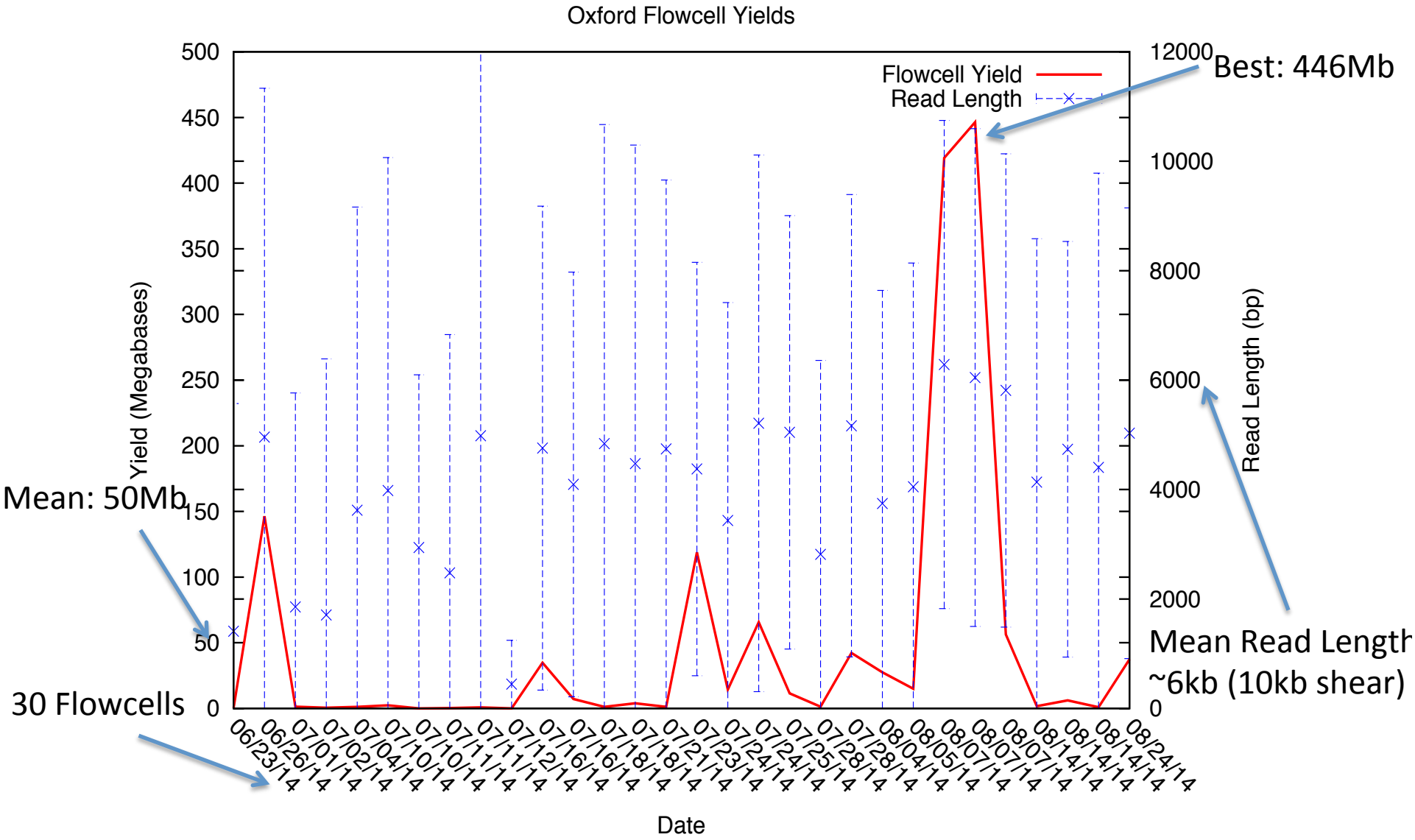
- Hidden Markov model
- Only four options per transition
- Pore type = distinct kmer length



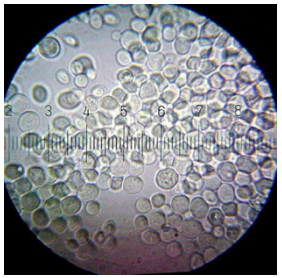
- Form probabilistic path through measured states currents and transitions
  - e.g. Viterbi algorithm

Basecalling currently performed at Amazon with frequent updates to algorithm

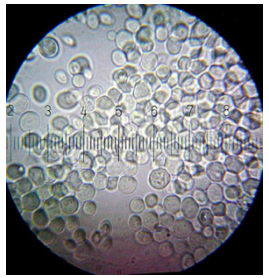
Our Data - Yeast W303

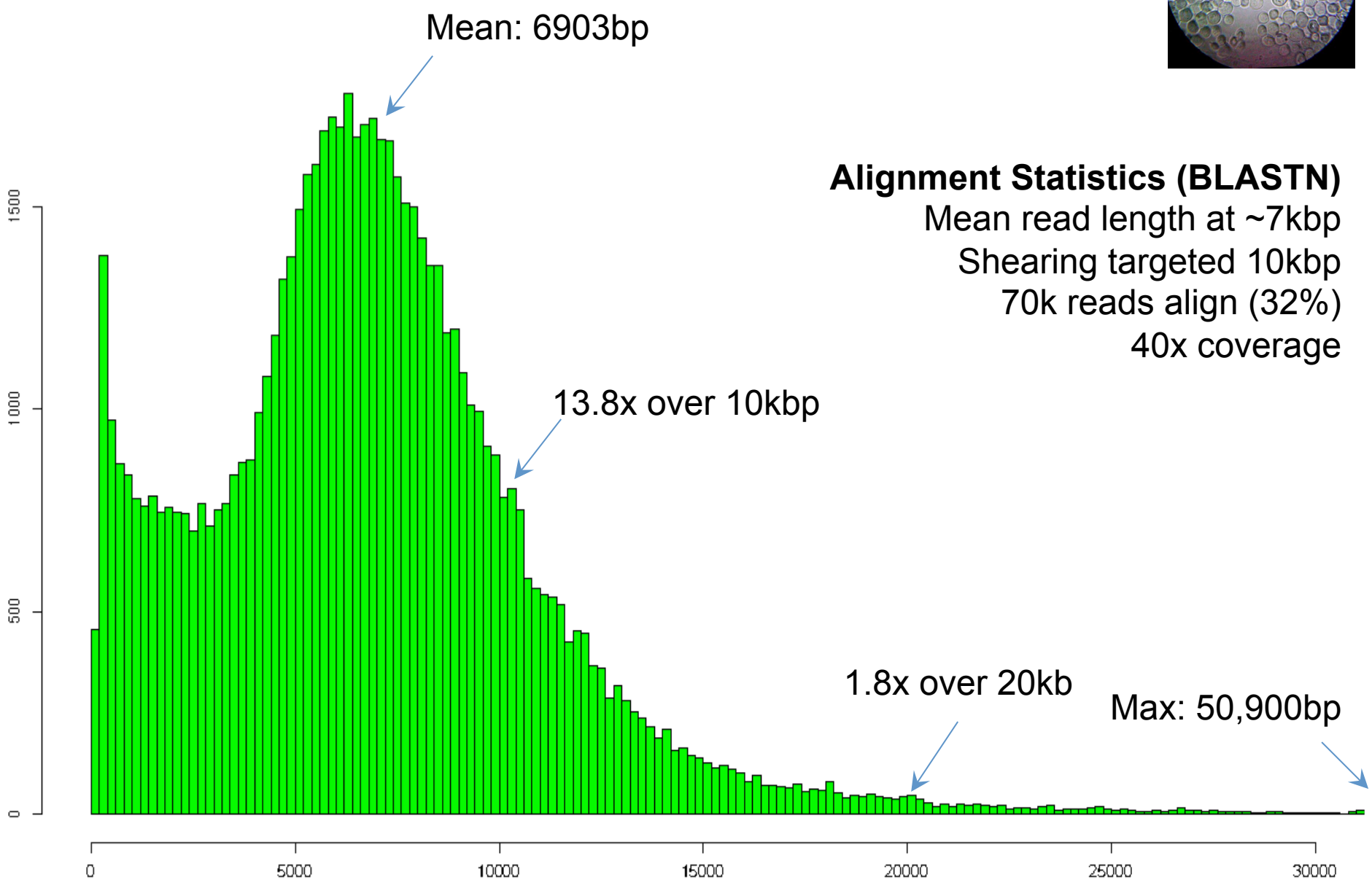


Nanopore Readlengths



Nanopore Alignments





Alignment Statistics (BLASTN)

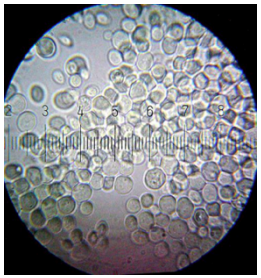
Mean read length at ~7kbp

Shearing targeted 10kbp

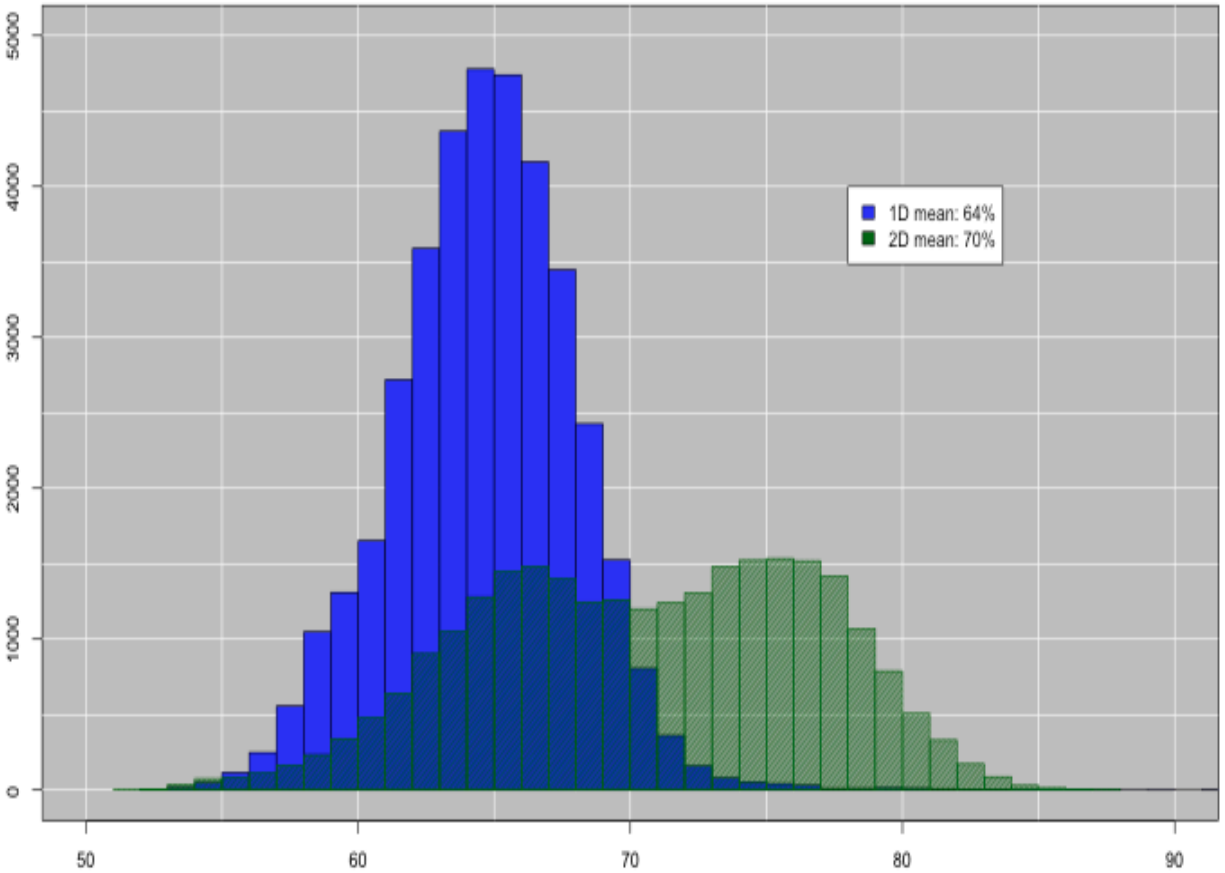
70k reads align (32%)

40x coverage

Nanopore Accuracy



Alignment Quality (BLASTN)
Of reads that align, average ~64% identity
"2D base-calling" improves to ~70% identity

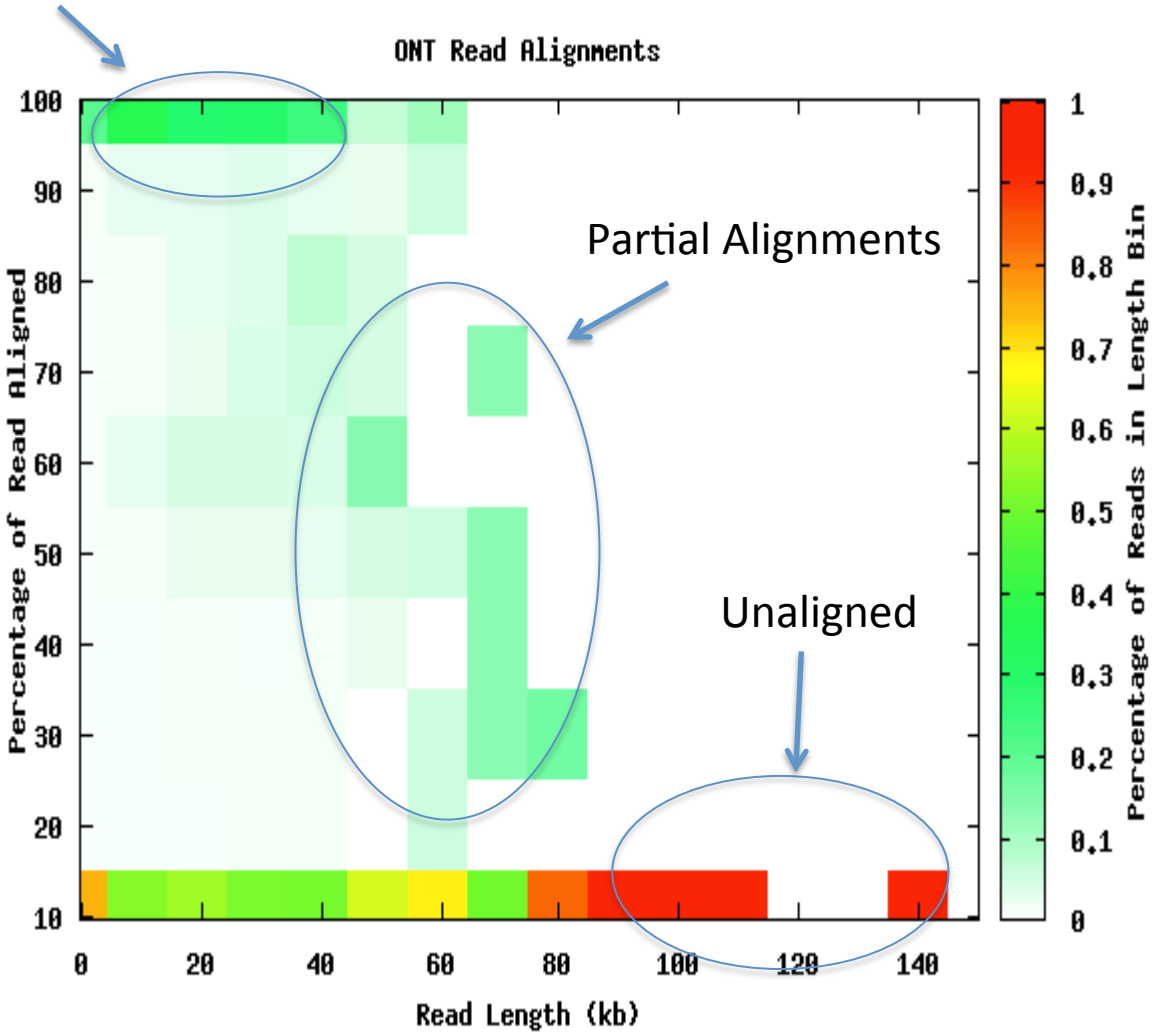


57% Mismatches
32% Deletions
11% Insertions

Nanopore Alignment Summary

32% of the data map using BLASTN

Full Length Alignments



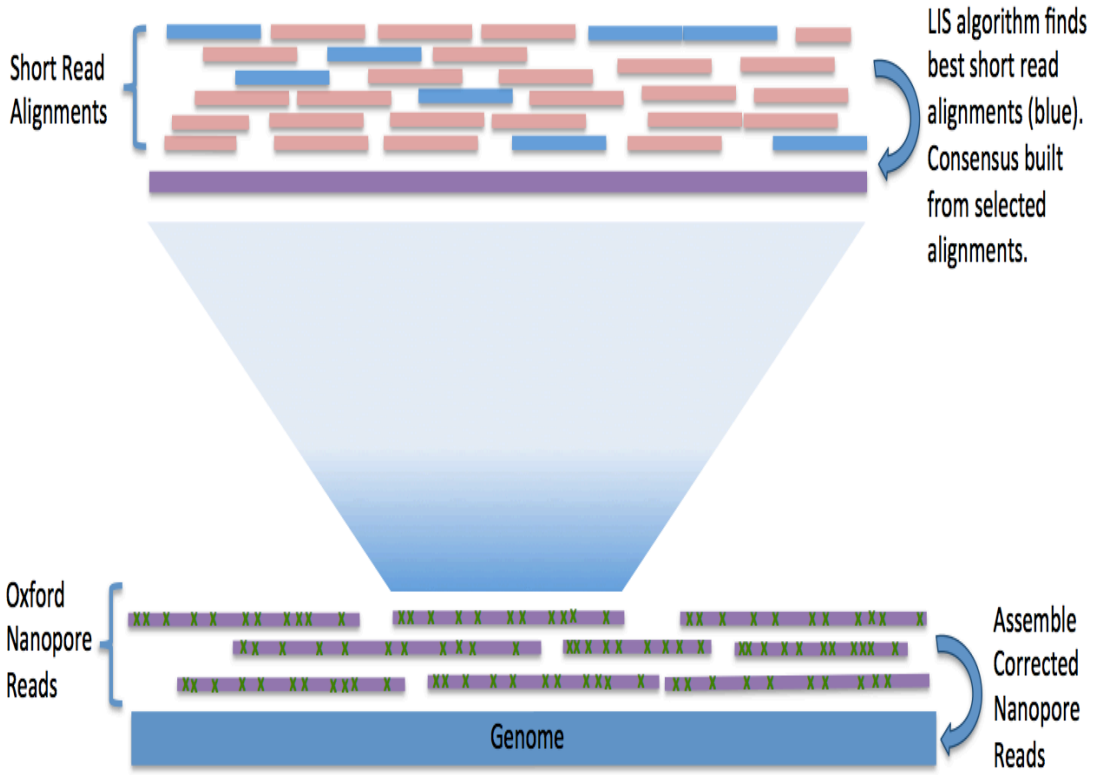
NanoCorr: Nanopore-Illumina Hybrid Error Correction

https://github.com/jgurtowski/nanocorr

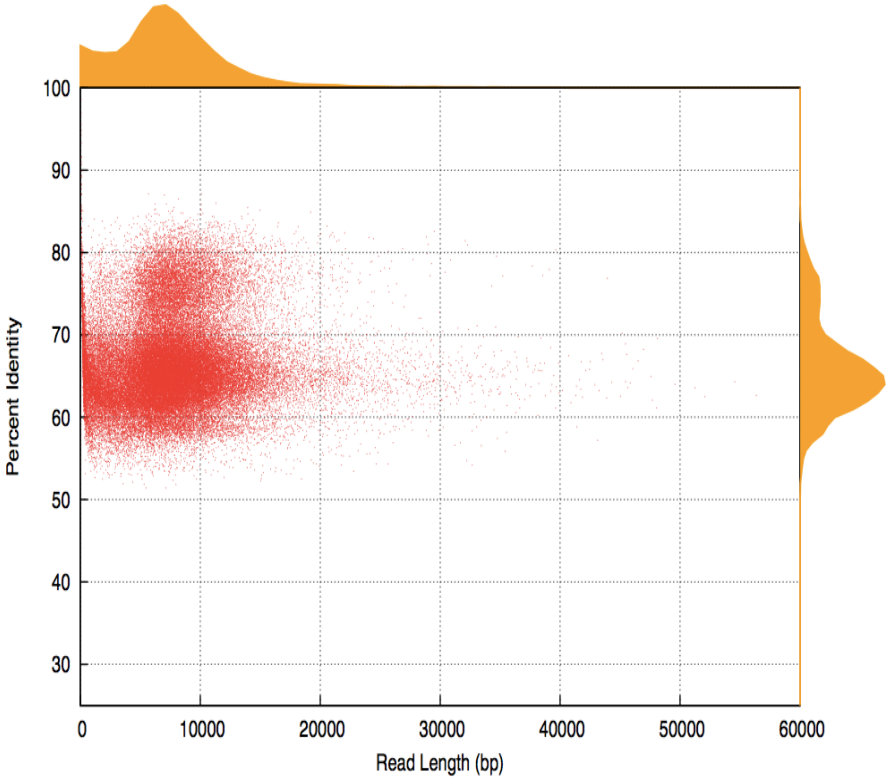
1. BLAST Miseq reads to all raw Oxford Nanopore reads

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

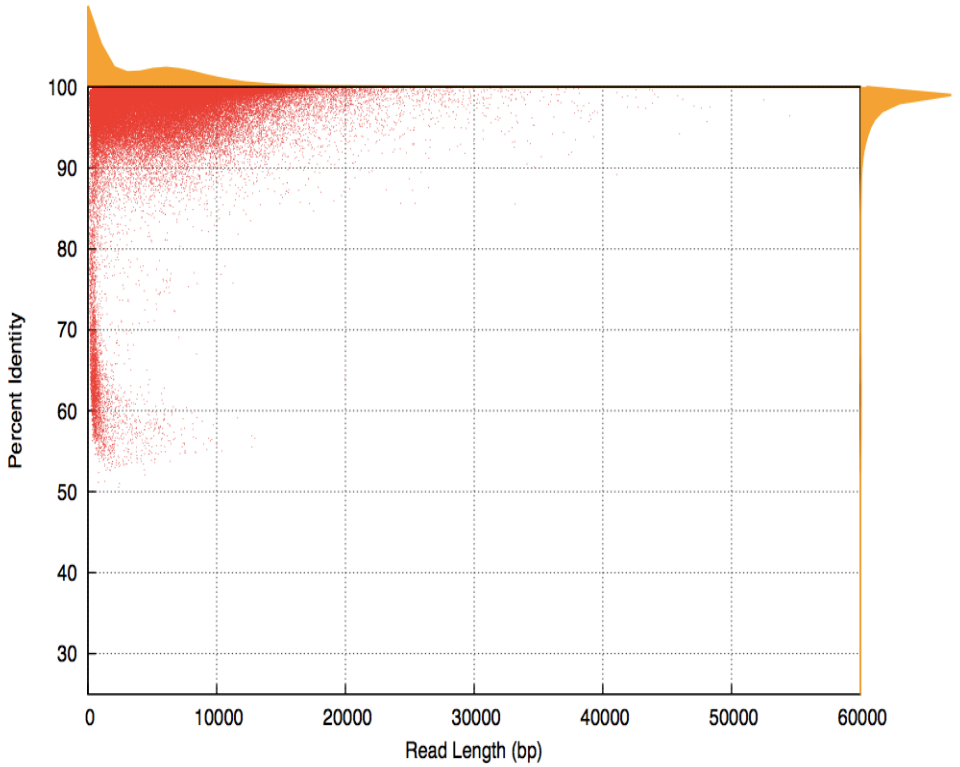
3. Compute consensus of each Oxford Nanopore read
    1. Currently using Pacbio's pbdagcon



Nanocorr correction pipeline significantly improves read identity



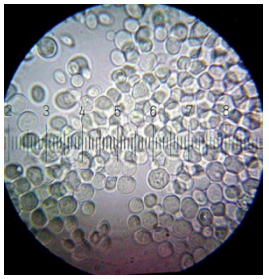
Before



After

Percent identity versus read length before and after nanocorr correction

Long Read Assembly



S288C Reference sequence

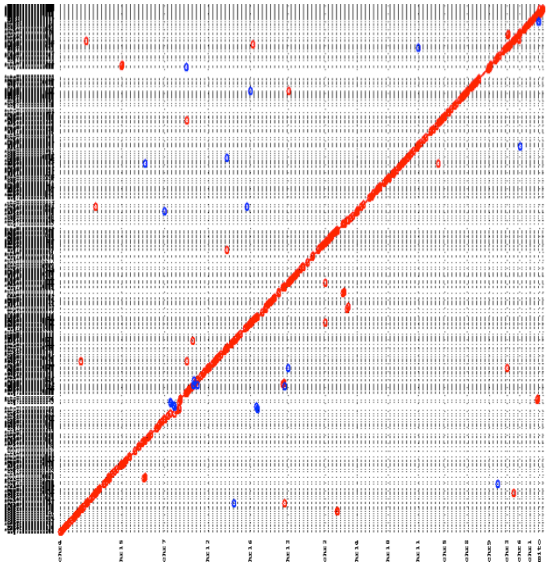
- 12.1Mbp; 16 chromo + mitochondria; N50: 924kbp

Illumina MiSeq

30x, 300bp PE (Flashed)

Celera Assembler

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

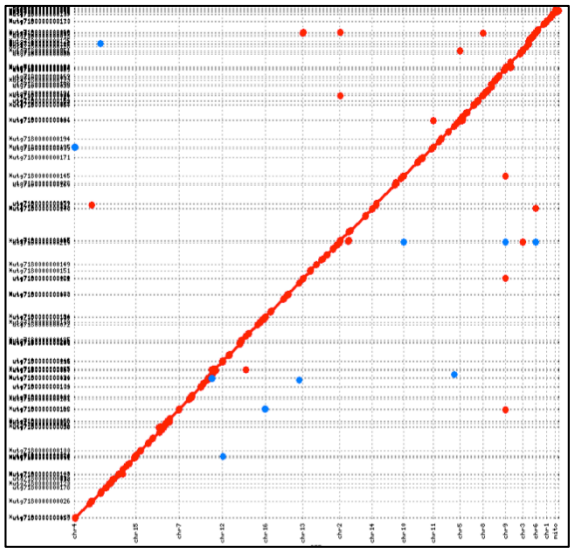


Oxford Nanopore

30x corrected reads > 6kb

NanoCorr + Celera Assembler

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

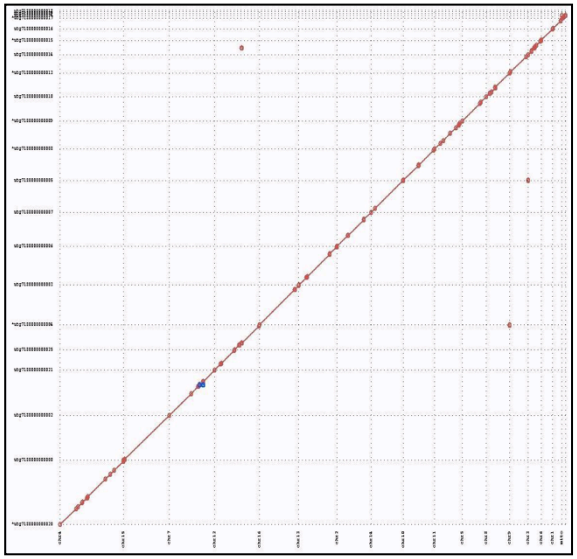


Pacific Biosciences

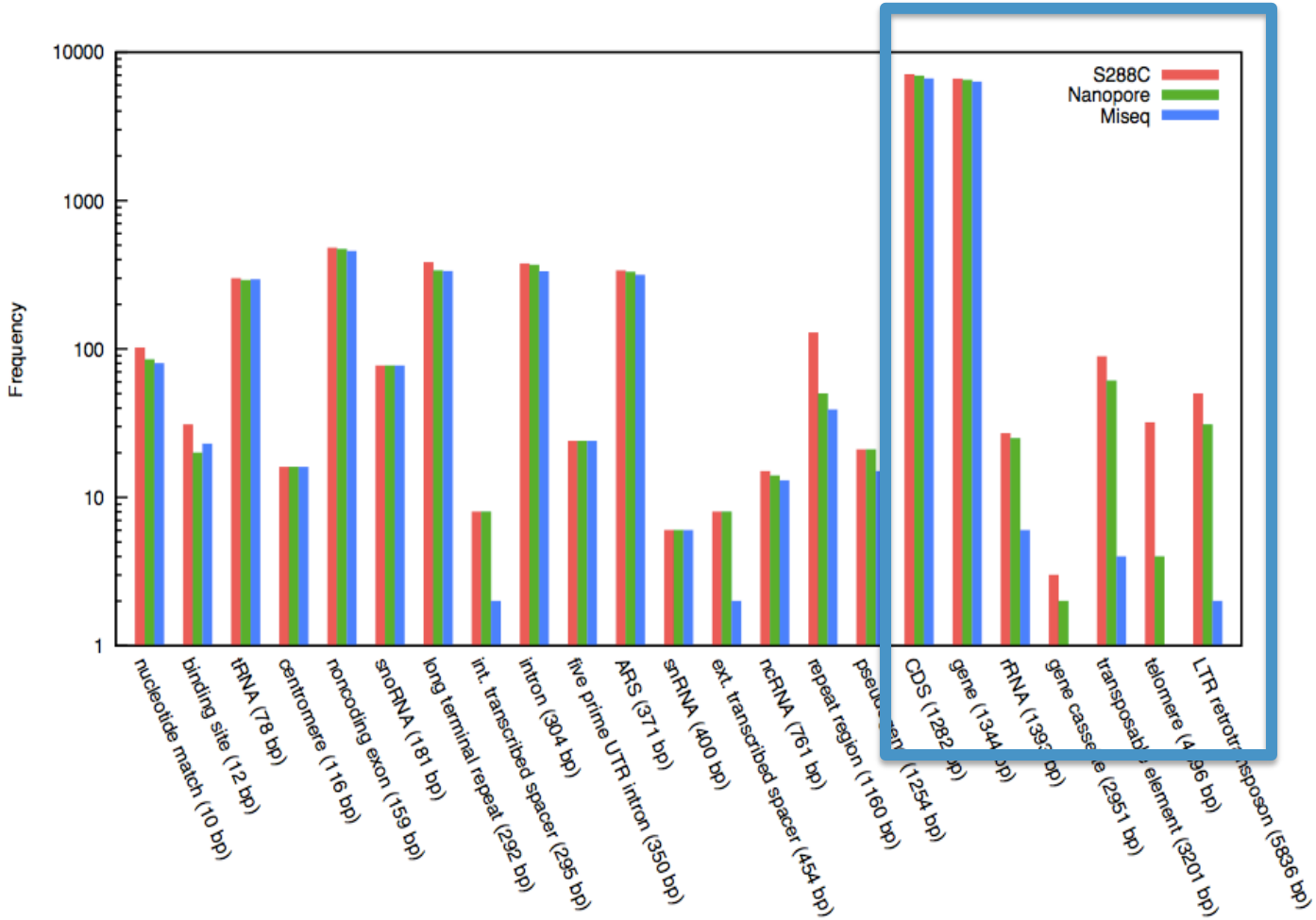
25x corrected reads > 10kb

HGAP + Celera Assembler

- 21 non-redundant contigs
- N50: 811kb >99.8% id

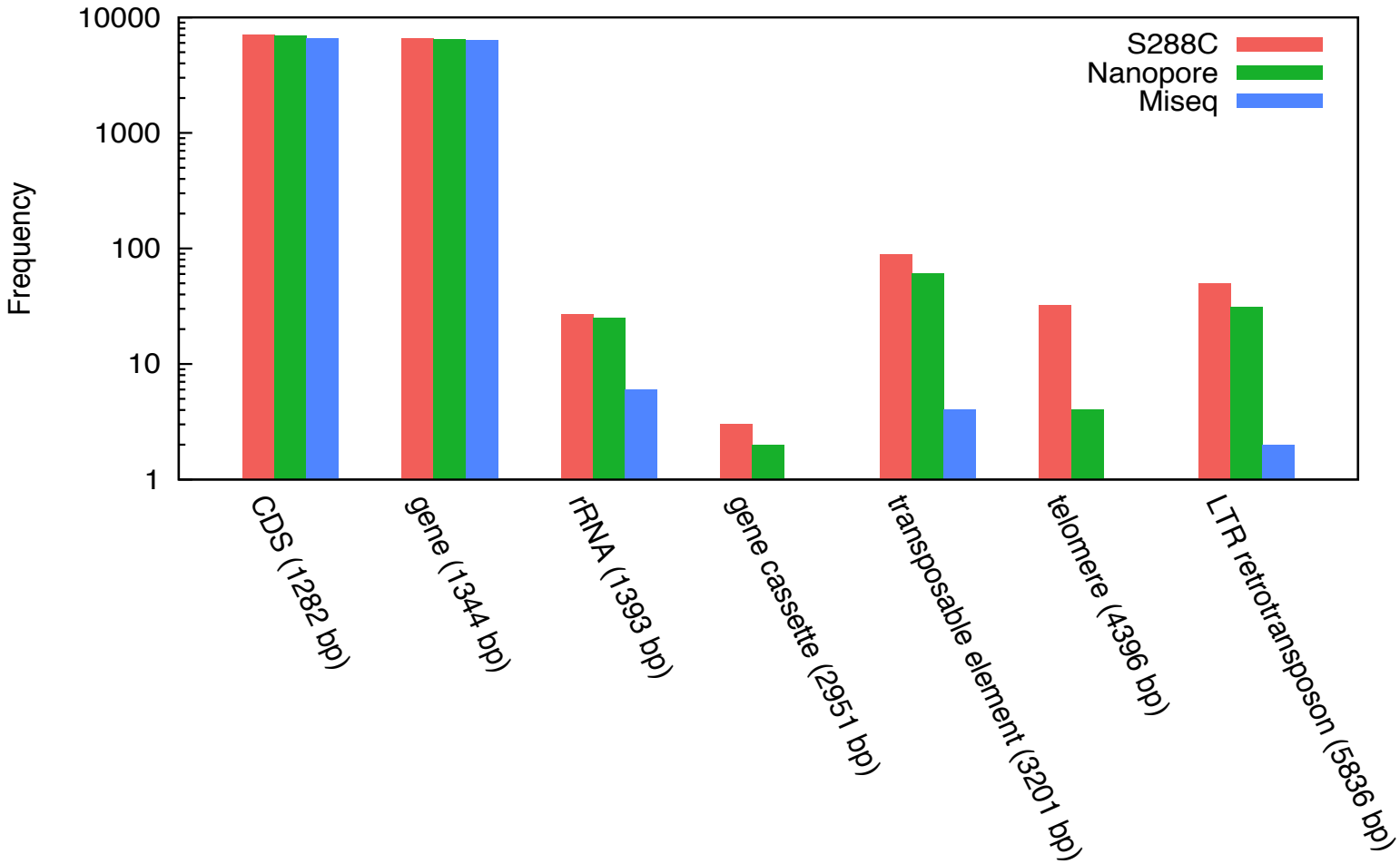


An assembly generated from Oxford Nanopore long reads is better able to identify genomic features

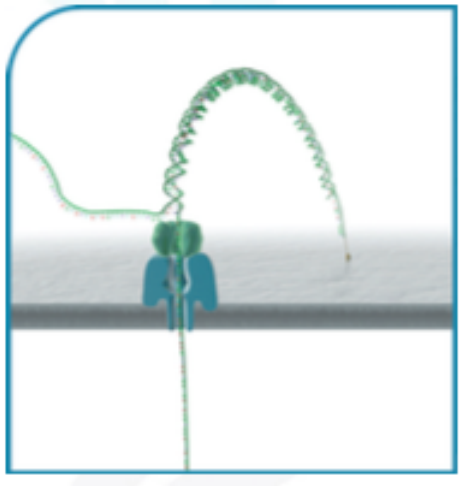


- S288C is an extremely high quality reference
- In virtually all cases the Oxford estimate of the frequncy of a genomic feature is closer to S288C than data generated by miSeq
- In some cases (gene cassette, telomere) the miSeq is completely unable to detect features

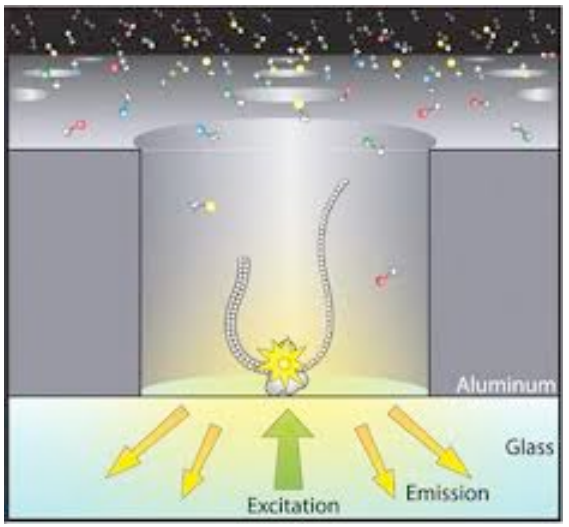
ONT Assembly Completeness



Summary



1. New Single Molecule Sequencing Technologies
2. Produce very long reads
3. Have High Error rate -> Error Correction
4. Long reads Produce great assemblies, far better than short read technologies -> repeat resolution



Future of Oxford Nanopore

PromethION Setup

PromethION is a standalone benchtop instrument that includes substantial on-board computing to enable very high throughput real-time analyses. It is compatible with the cloud-based analysis service from Metrichor.

PromethION contains docking for 48 flow cells. Each flow cell contains a nanopore sensor array enabling 3,000 nanopores, so a total of 144,000 on the instrument. The sensor array interfaces **with an ASIC within the instrument** for signal processing. Each flow cell also allows for multiple samples to be processed separately.

Future for Pacbio

Sequel System: high-throughput, cost-effective access to SMRT Sequencing

The Sequel System is ideal for projects such as rapidly and cost-effectively generating high-quality whole genome *de novo* assemblies.

Learn more about the Sequel System.

Acknowledgements

Cold Spring Harbor Laboratory

Michael Schatz

Dick McCombie

Sara Goodwin

Schatz Lab

- Tyler Garvin
- Han Fang
- Hayan Lee
- Maria Nattestad
- Srividya Ramakrishnan

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