CSH Cold Spring Harbor Laboratory

# 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
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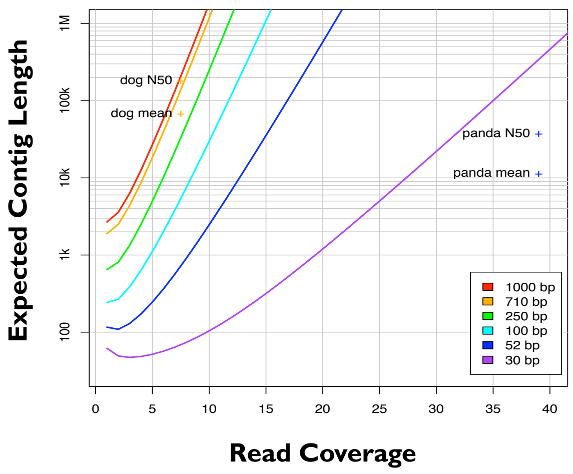
4. Summary

# Ingredients for a good assembly

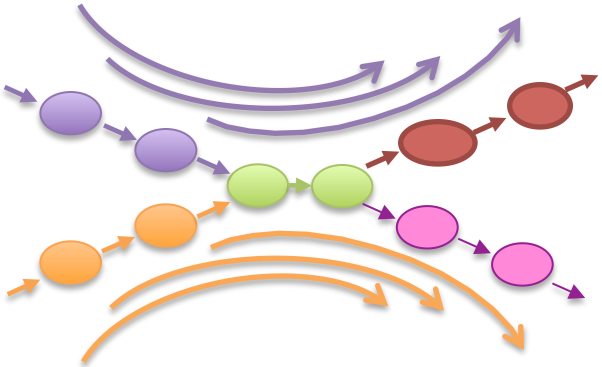
## Coverage



***High coverage is required***

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

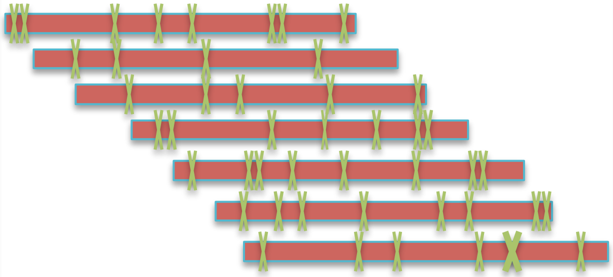
## Read Length



***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

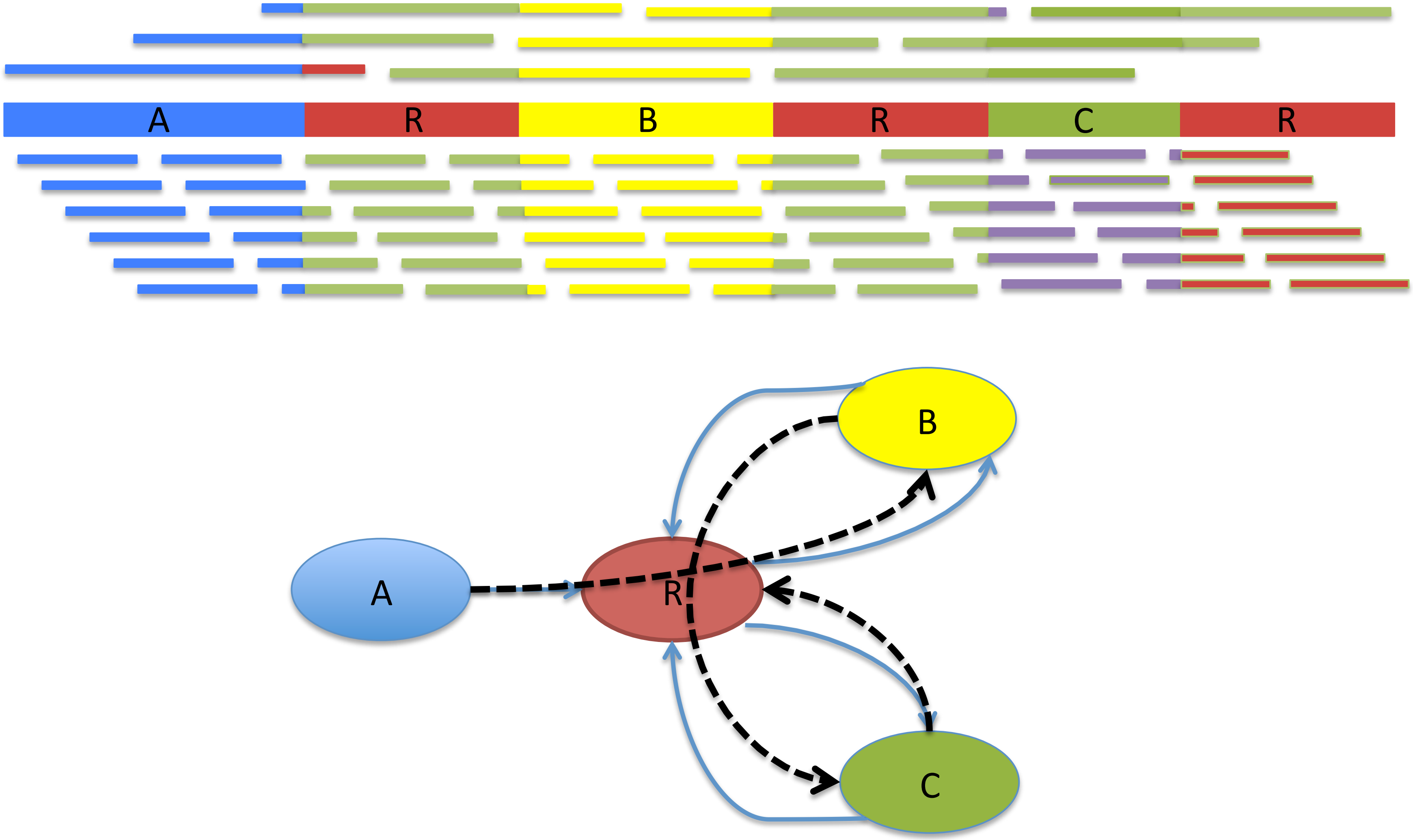


***Errors obscure overlaps***

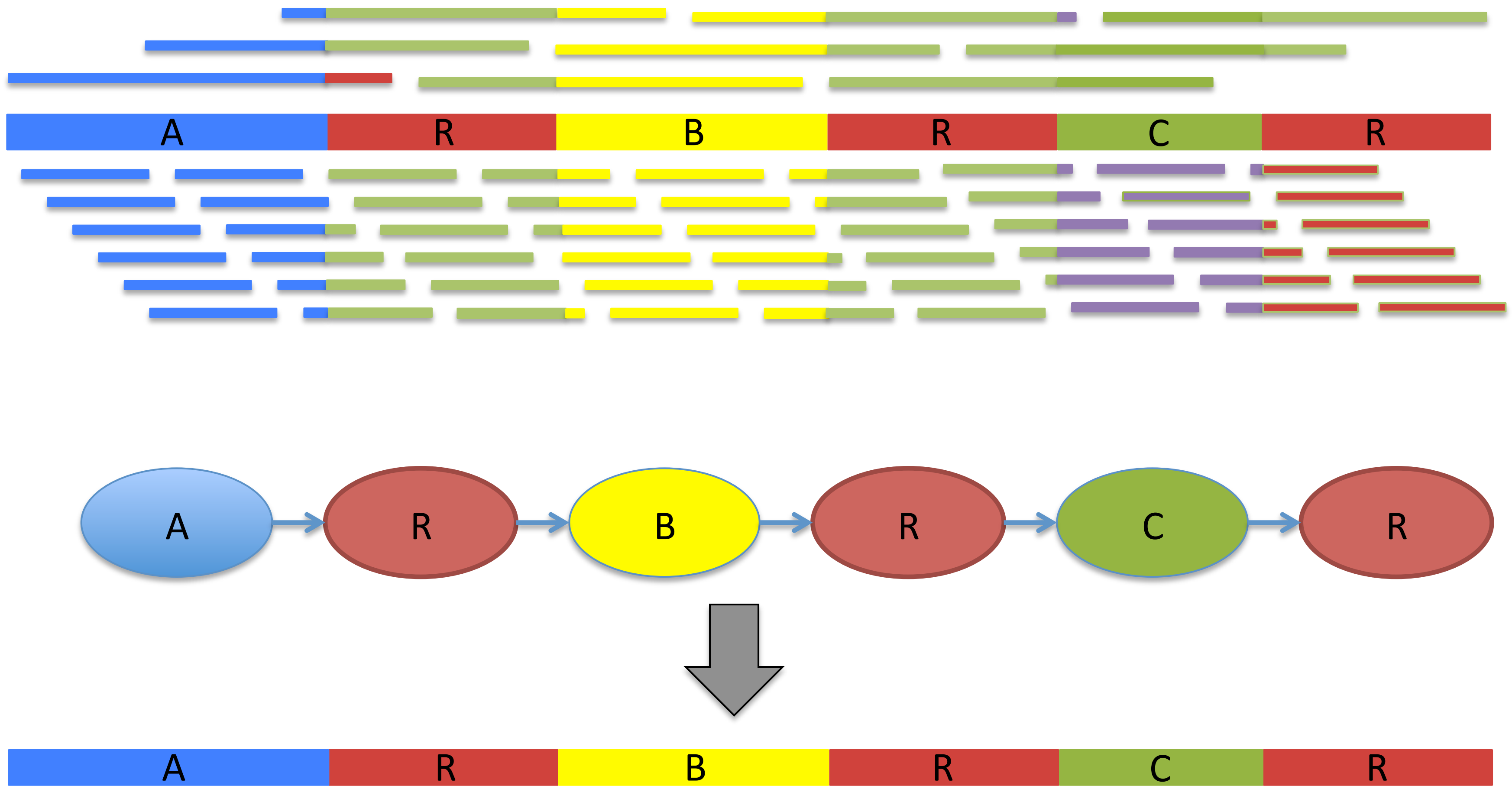
- – Reads are assembled by finding kmers shared in pair of reads
- – High error rate requires very short seeds, increasing complexity and forming assembly hairballs

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

# Assembly Complexity



# Assembly Complexity

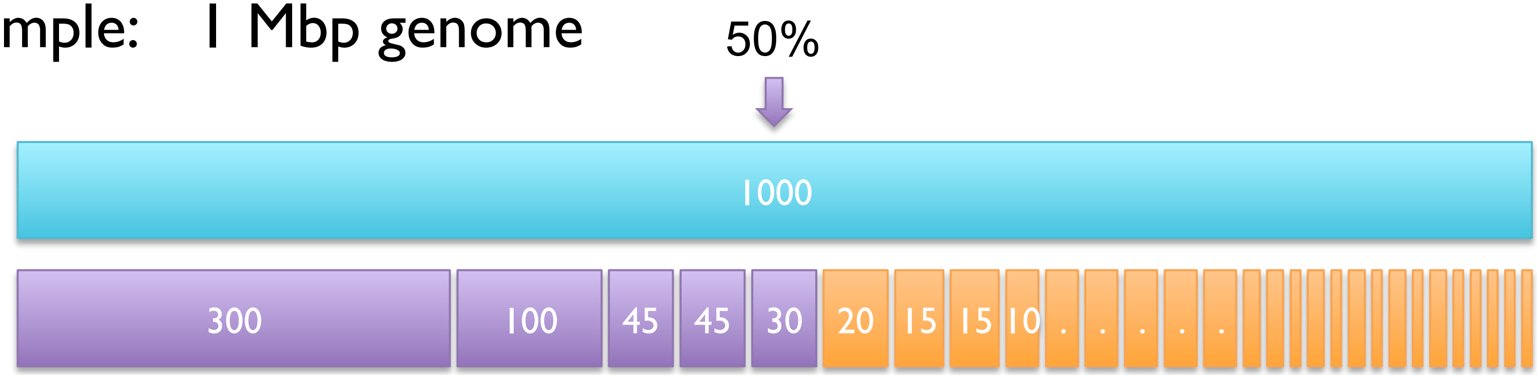


**The advantages of SMRT sequencing**
Roberts, RJ, Carneiro, MO, Schatz, MC (2013) *Genome Biology.* 14:405

# 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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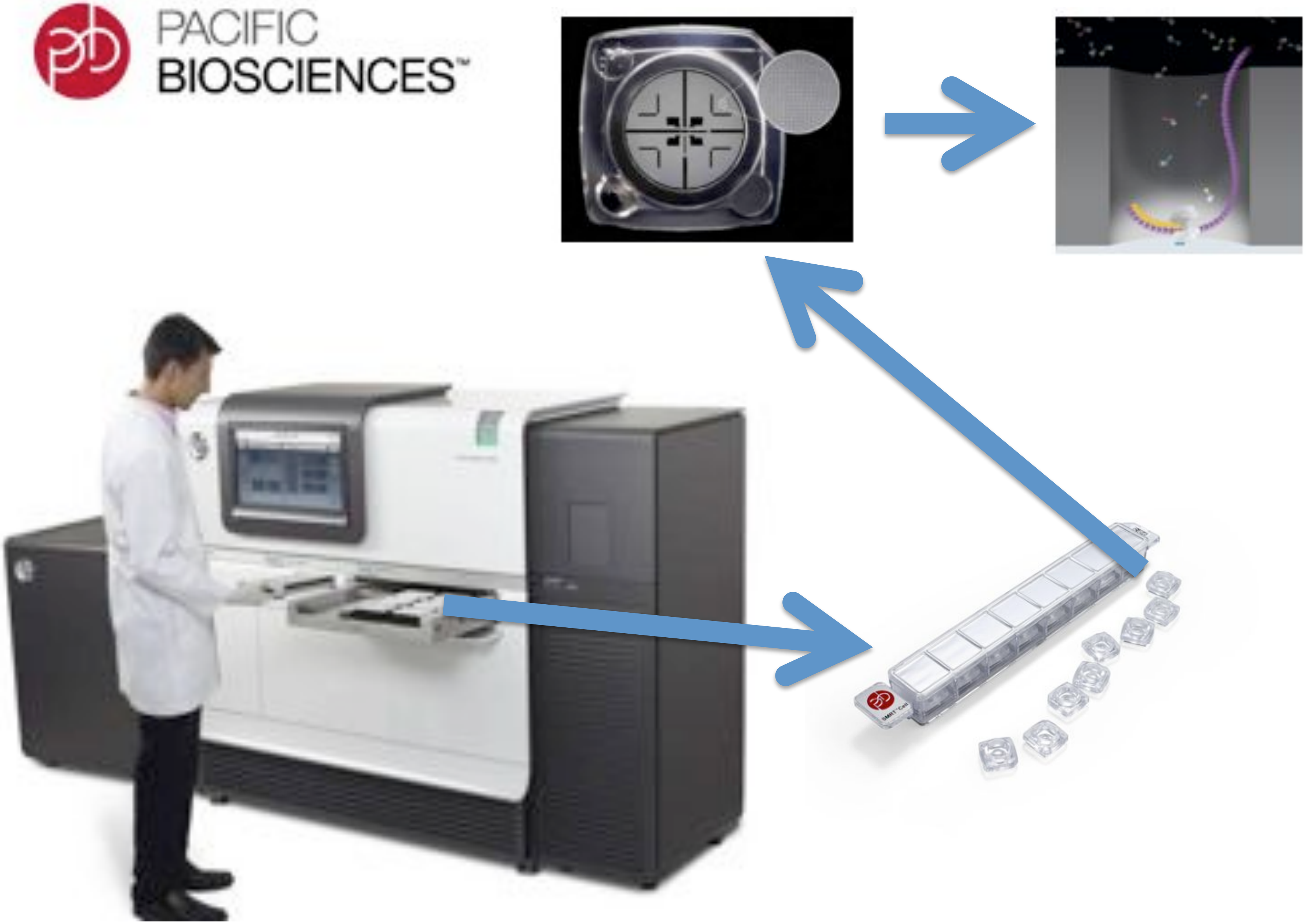
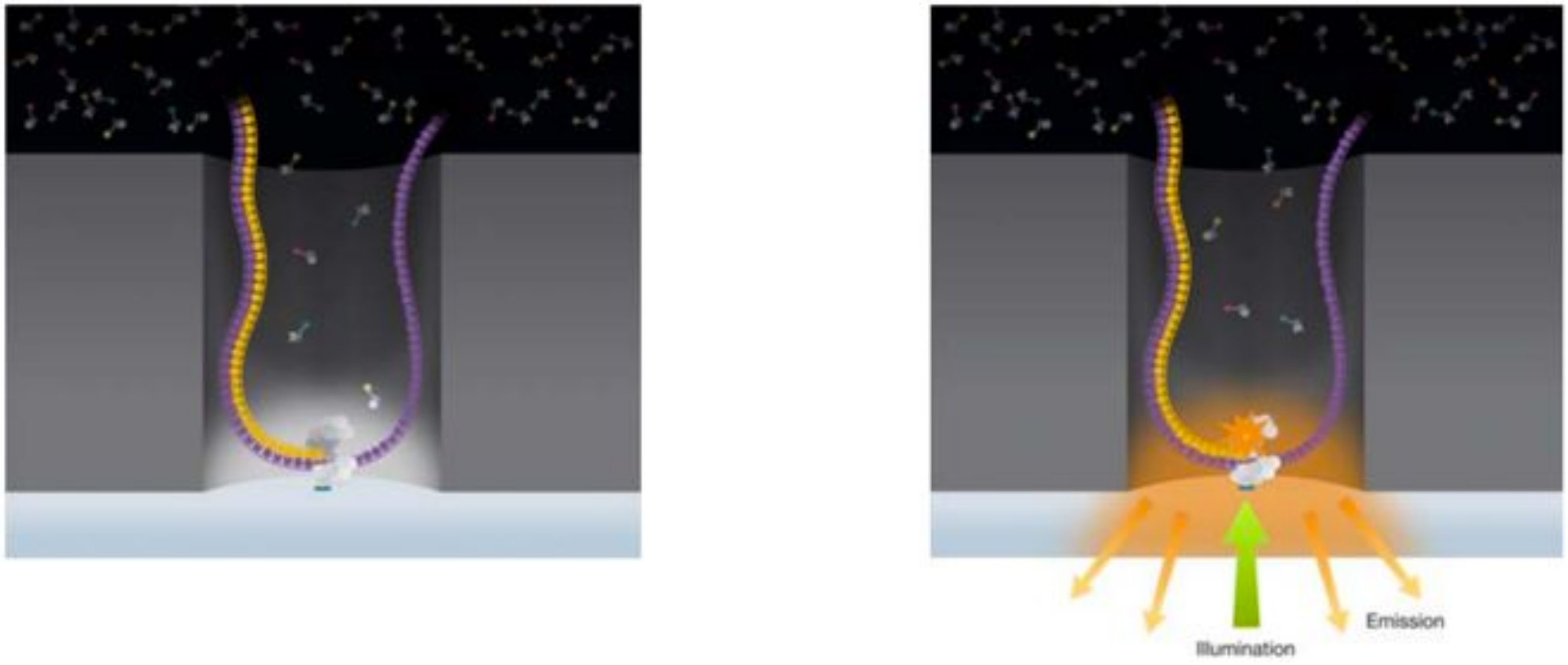
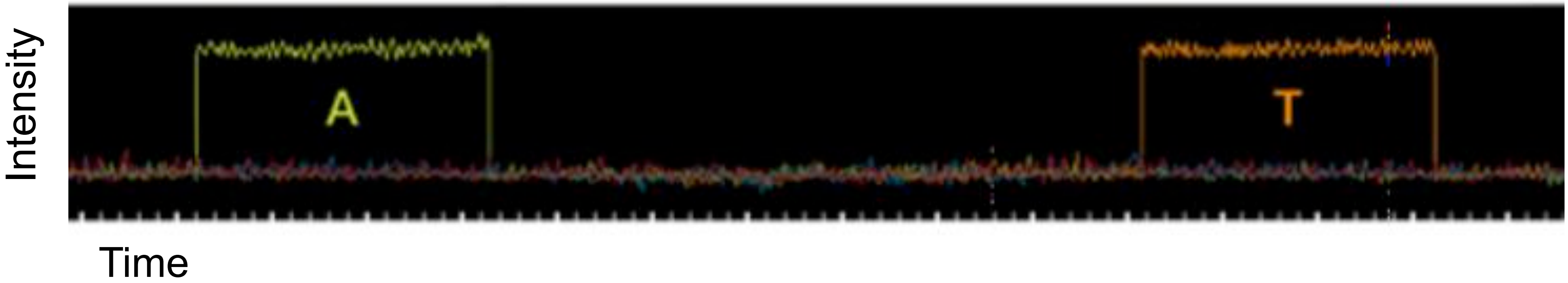


# SMRT Sequencing

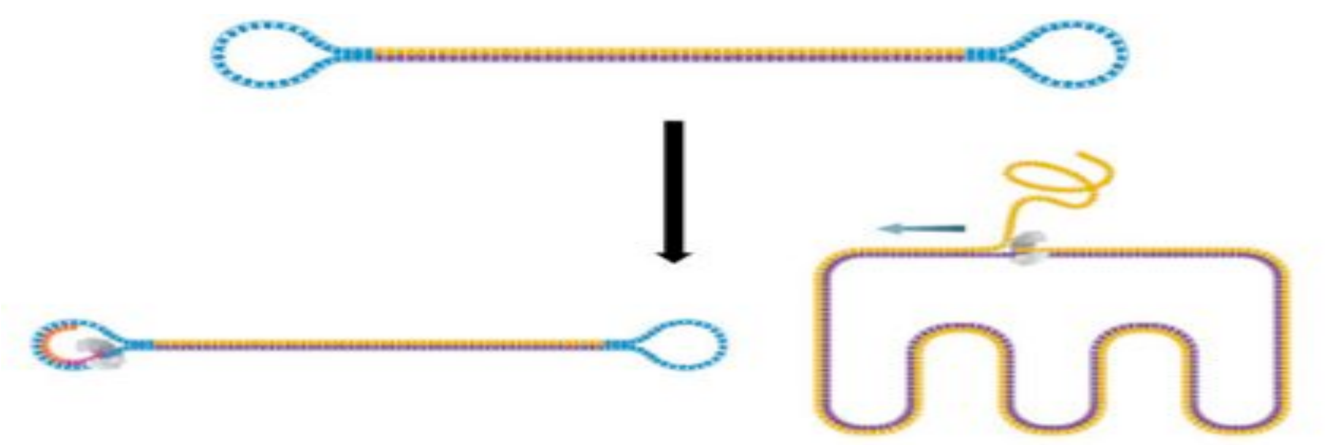
Imaging of fluorescently phospholinked labeled nucleotides as they are incorporated by a polymerase anchored to a Zero-Mode Waveguide (ZMW).





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

# SMRT Read Types

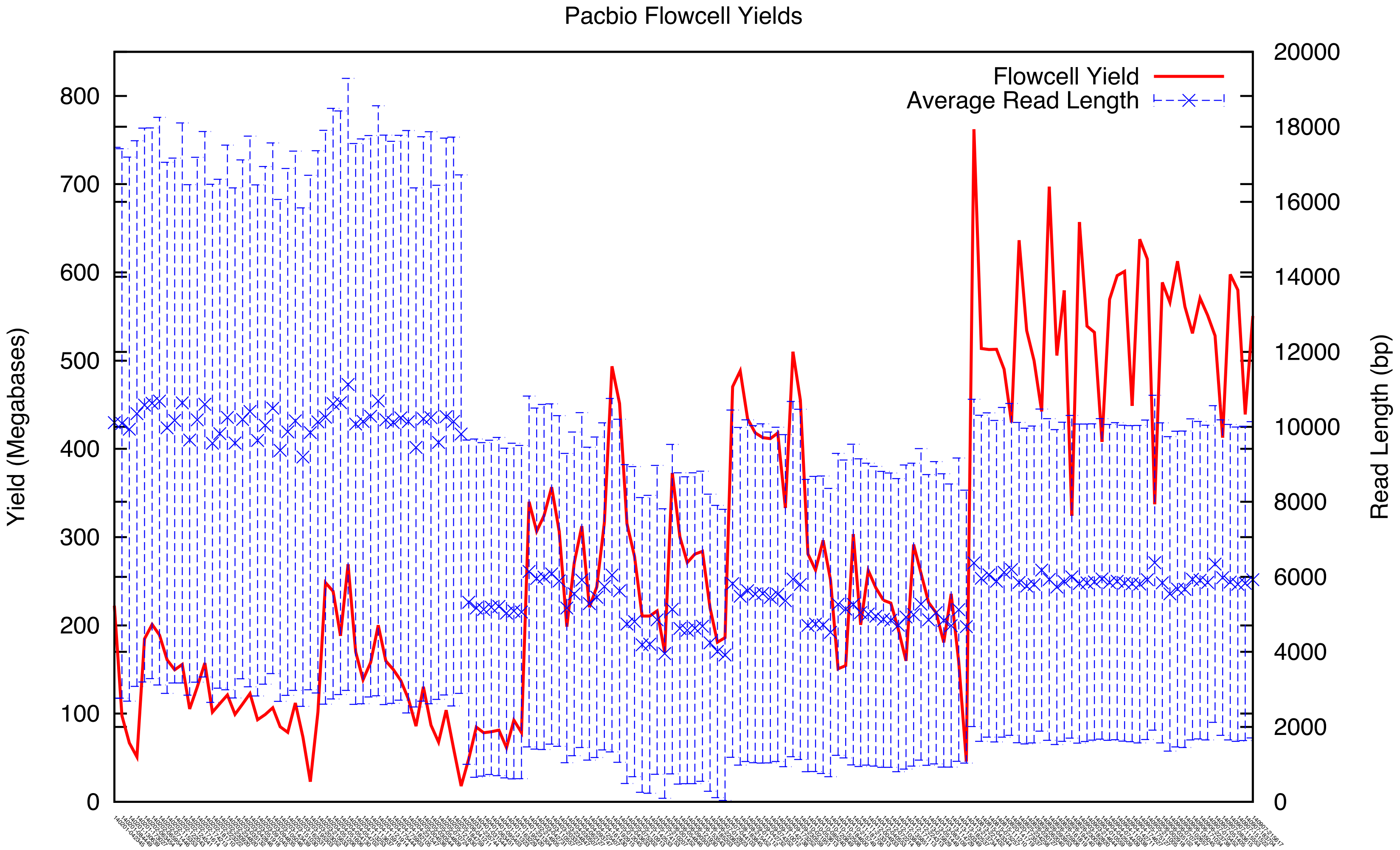


- ***Standard sequencing***
  - – Long inserts so that the polymerase can synthesize along a single strand

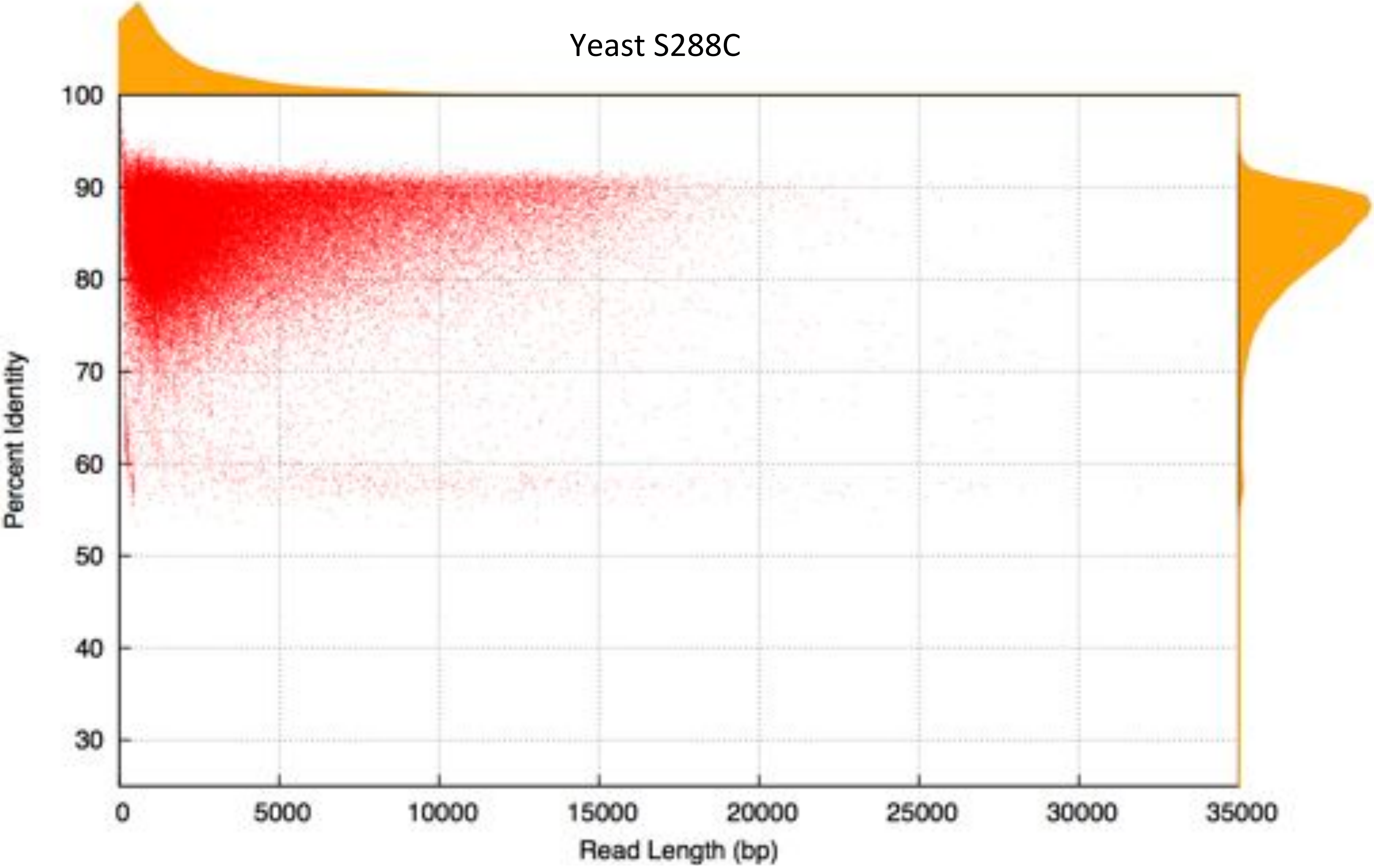
- ***Circular consensus sequencing***
  - – Short inserts, so polymerase can continue around the entire SMRTbell multiple times and generate multiple sub-reads from the same single molecule.
  - – Barbell sequence: ATCTCTCTCttttcctcctcctccgttgttgttgttGAGAGAGAT

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

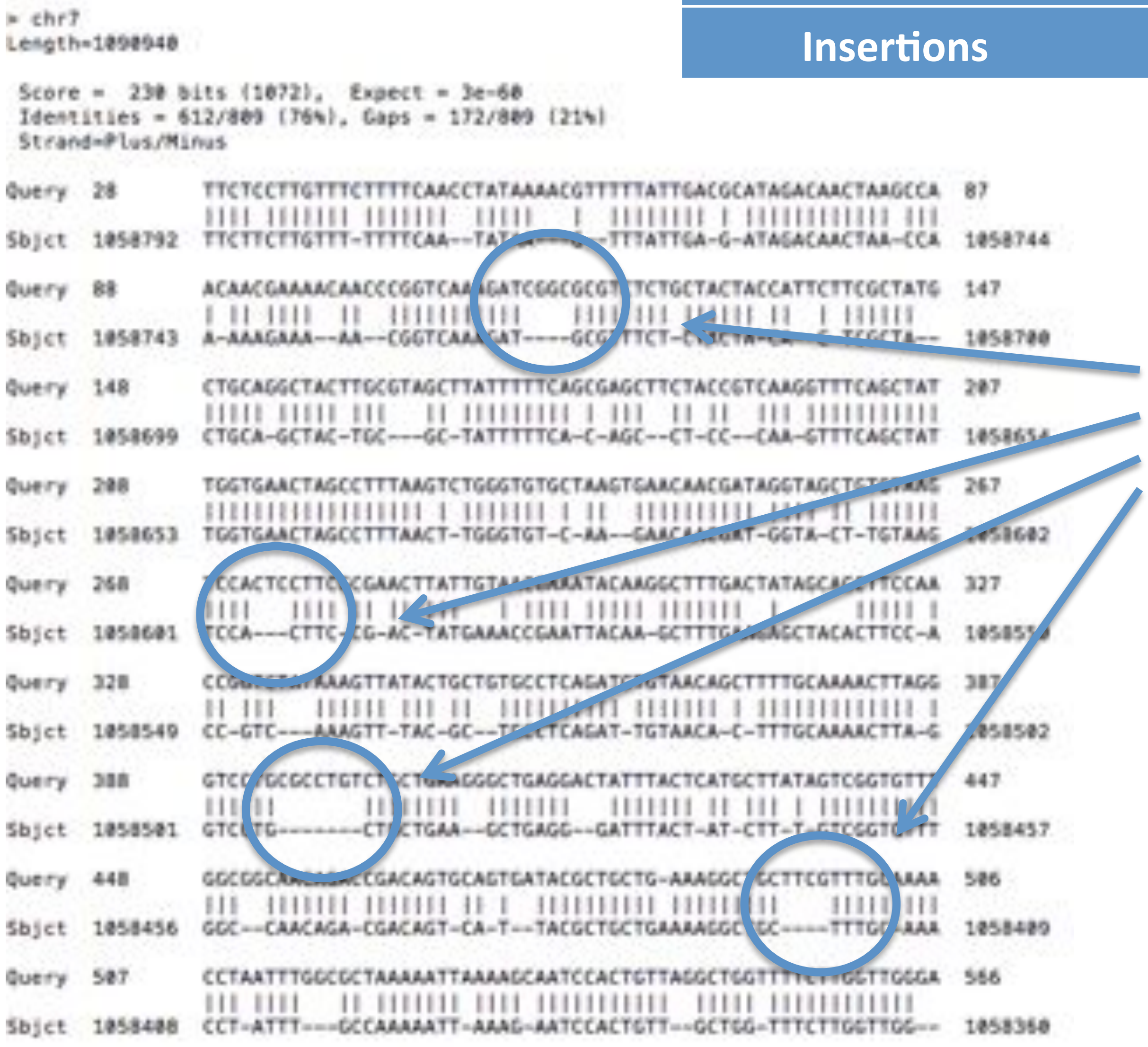
# Example Flowcell Yields
# Rice Strain IR64 (Feb – Sep 2014)



# Read Length Distribution and Identity



|  |  |
|---|---|
| **Average Error Rate** | 16% |
| **Mismatches** | 30.0% |
| **Deletions** | 11.5% |
| **Insertions** | 58.5% |

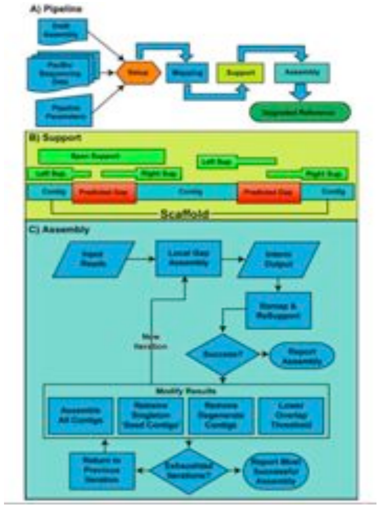


```
> chr7
Length=1090940

 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--TATCA---G--TTTATTGA-G-ATAGACAACTAA-CCA  1058744

Query  88       ACAACGAAAACAACCCGGTCAAAGATCGGCGCGTTTCTGCTACTACCATTCTTCGCTATG  147
                | || ||||  ||  ||||||||||||     |||| ||| |||||| ||   | ||||||
Sbjct  1058743  A-AAAGAAA--AA--CGGTCAAAGAT----GCGTTTCT-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      TCCACTCCTTCCCGAACTTATTGTAAGGAAATACAAGGCTTTGACTATAGCACGTTCCAA  327
                ||||    |||| || |||||     | |||| ||||| ||||||||  |        ||||| |
Sbjct  1058601  TCCA---CTTC-CG-AC-TATGAAACCGAATTACAA-GCTTTGAAGAGCTACACTTCC-A  1058550

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

Query  388      GTCCTGCGCCTGTCTGCTGAAGGGCTGAGGACTATTTACTCATGCTTATAGTCGGTGTTT  447
                ||||||        |||||||| ||||||||    |||||||| || ||| | ||||||||||
Sbjct  1058501  GTCCTG--------CTGCTGAA--GCTGAGG--GATTTACT-AT-CTT-T-GTCGGTGTTT  1058457

Query  448      GGCGGCAACAGAACGACAGTGCAGTGATACGCTGCTG-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

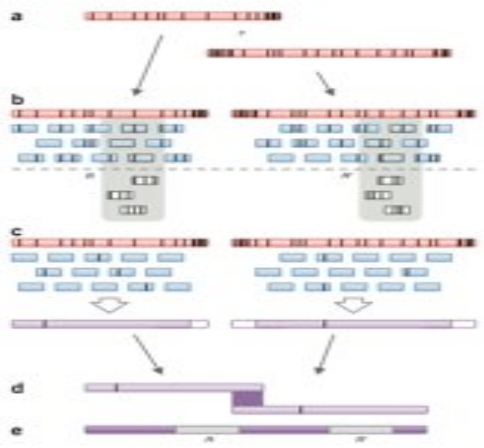
## PBJelly



**Gap Filling and Assembly Upgrade**

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

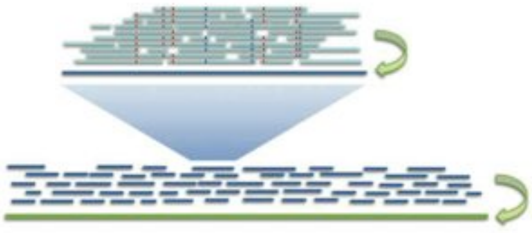
## PacBioToCA & ECTools



**Hybrid Error Correction**

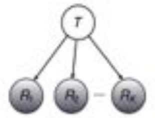
Koren, Schatz, *et al* (2012)
*Nature Biotechnology.* 30:693–700

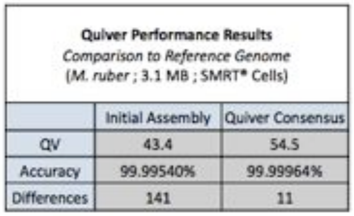
## HGAP & Quiver



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

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





**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**

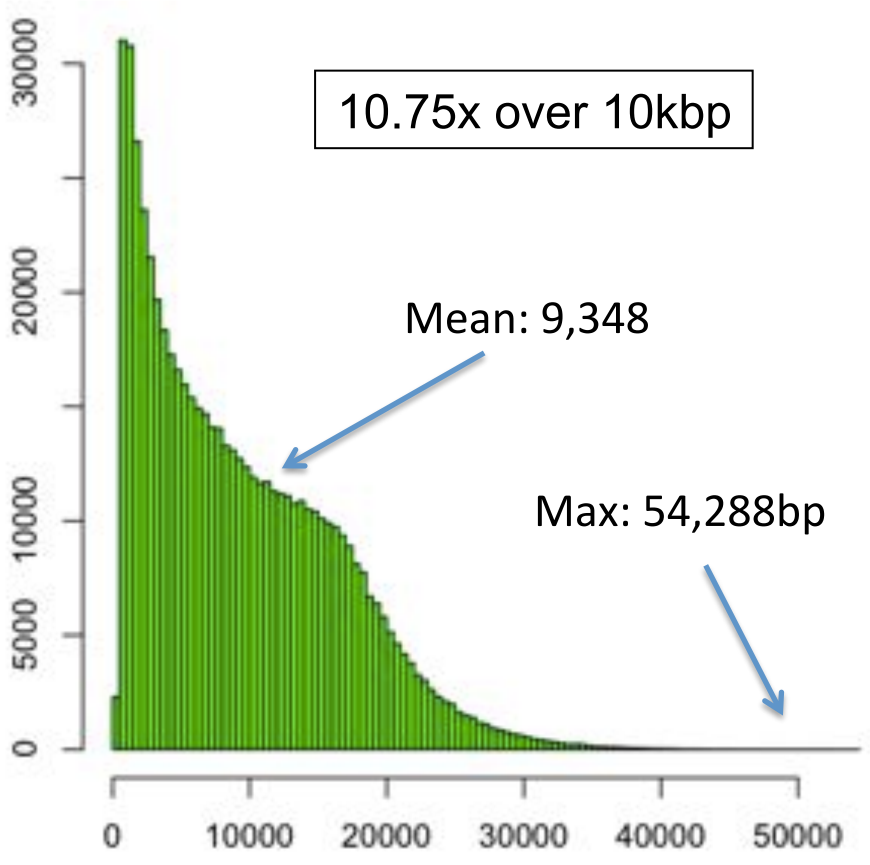
Chin *et al* (2013)
*Nature Methods.* 10:563–569

< 5x Long Read Coverage > 50x

# O. sativa pv Indica (IR64)

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

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



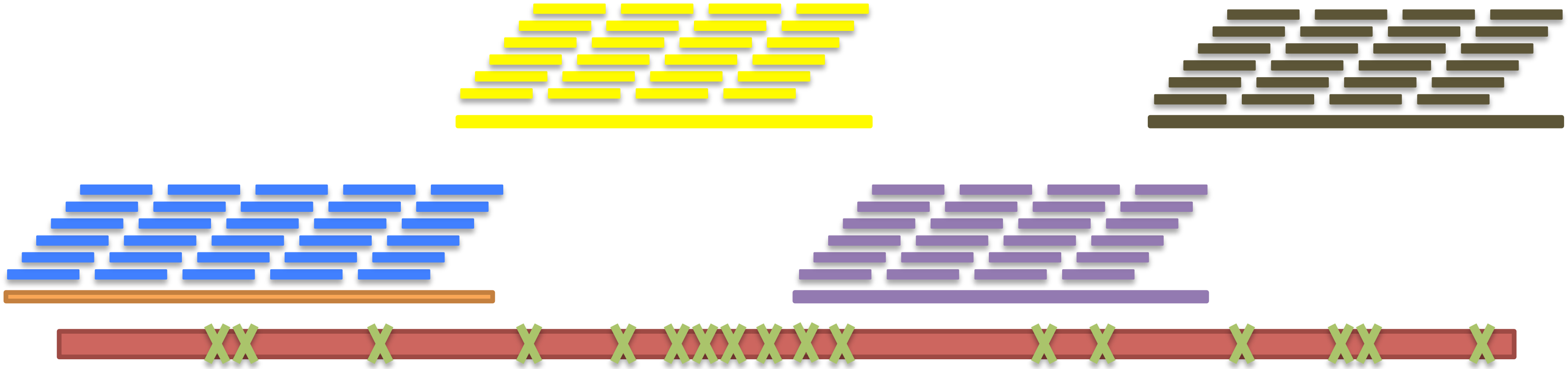
10.75x over 10kbp

Mean: 9,348

Max: 54,288bp

# ECTools: Error Correction with pre-assembled reads

https://github.com/jgurtowski/ectools



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

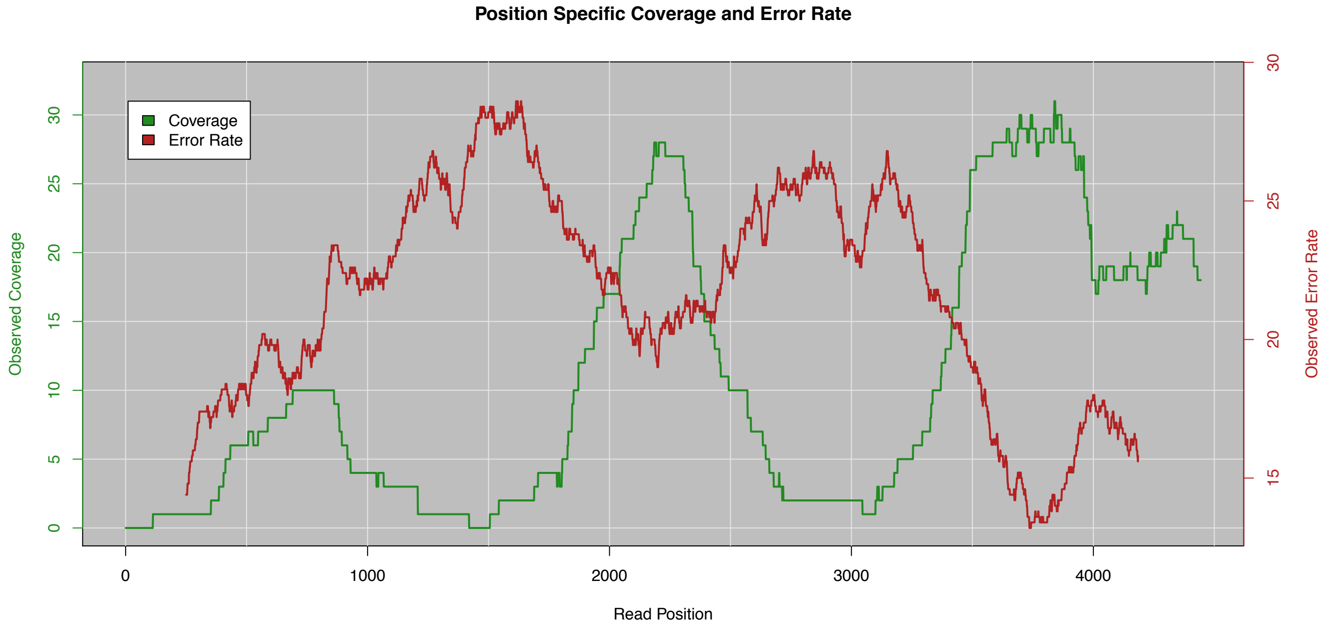
Can Help us overcome:

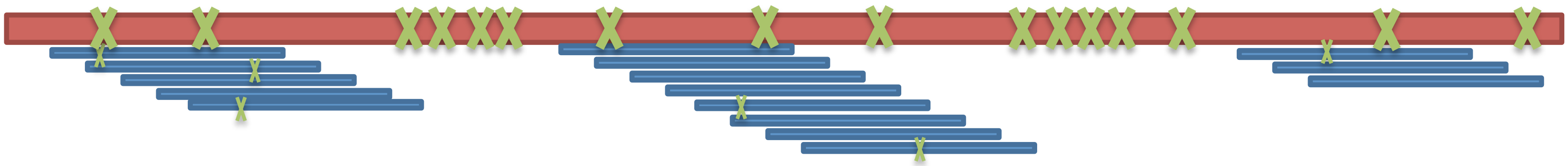
1. Error Dense Regions – Longer sequences have more seeds to match
2. Simple Repeats – Longer sequences easier to resolve

**However, cannot overcome Illumina coverage gaps & other biases**

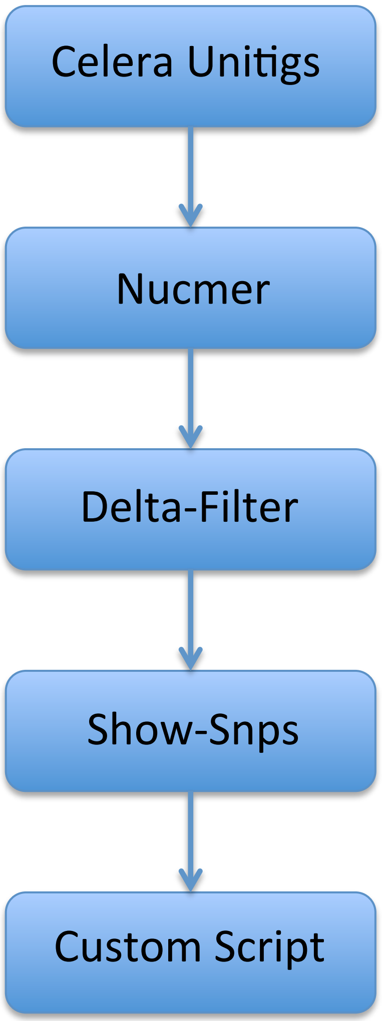
# Low Coverage Regions

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**


Position Specific Coverage and Error Rate



# ECTools Pipeline



Celera Unitigs

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

Nucmer

Align Unitigs to Pacbio Reads With Nucmer

Delta-Filter

Use Delta-Filter to Generate Unitig Layout

Show-Snps

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

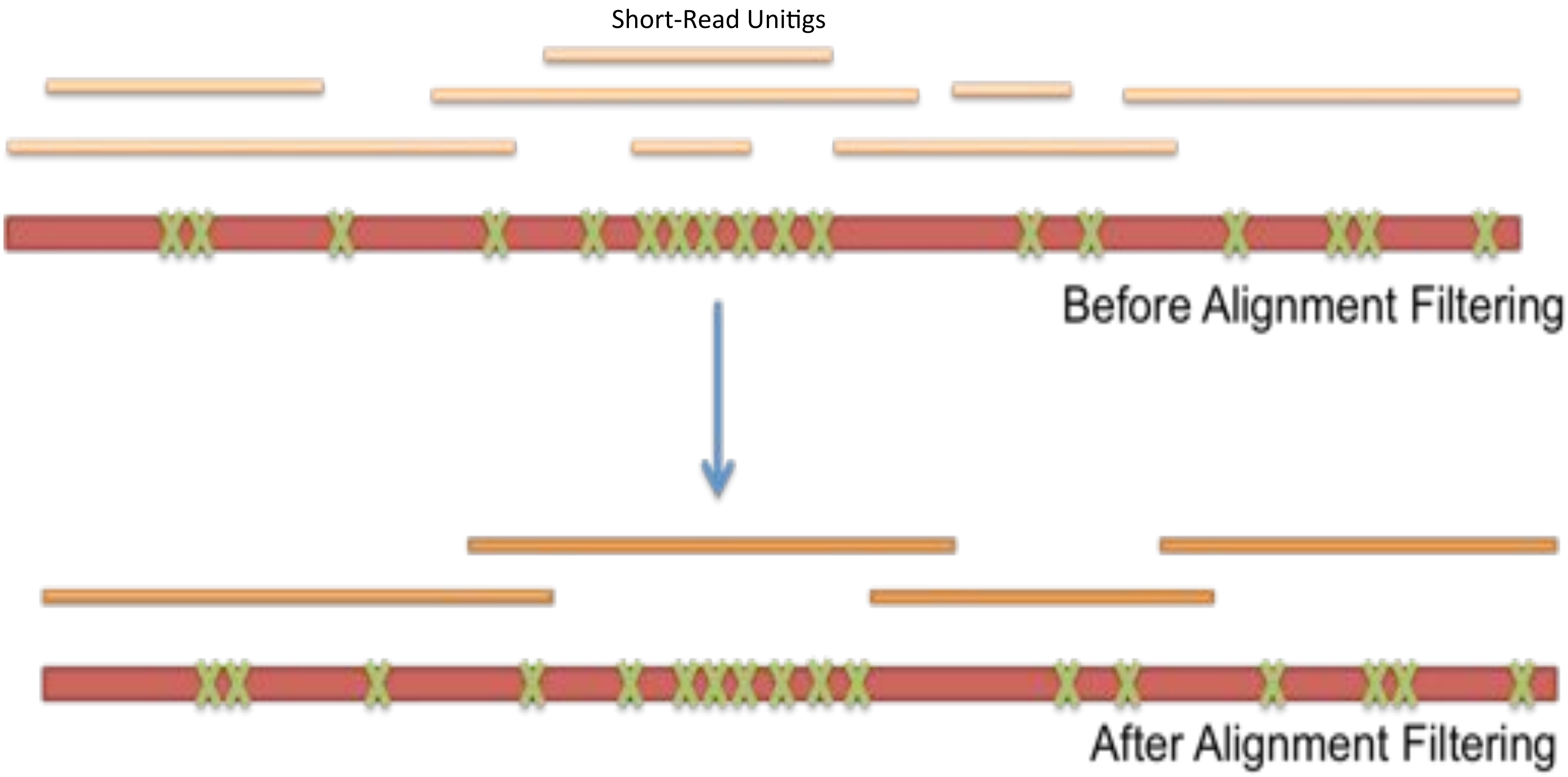
Custom Script

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



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Genome size: ~370 Mb

Chromosome N50: ~29.7 Mbp

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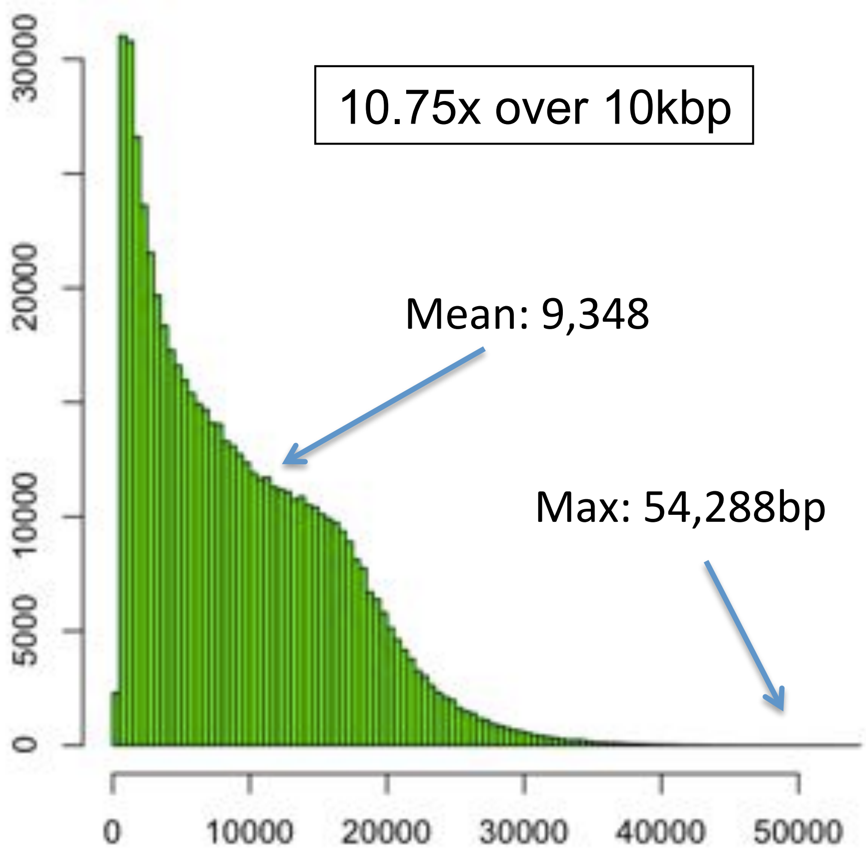
| **“ALLPATHS-recipe”**<br>50x 2x100bp @ 180<br>36x 2x50bp @ 2100<br>51x 2x50bp @ 4800 | 18,450 |
| MiSeq Fragments<br>25x 456bp<br>(3 runs 2x300 @ 450 FLASH) | 19,078 |
| PacbioToCA – 47 SMRTCells<br>10.7x @ 10kbp | 144,042 |
| ECTools - 47 SMRTCells<br>10.7x @ 10kbp | 272,137 |

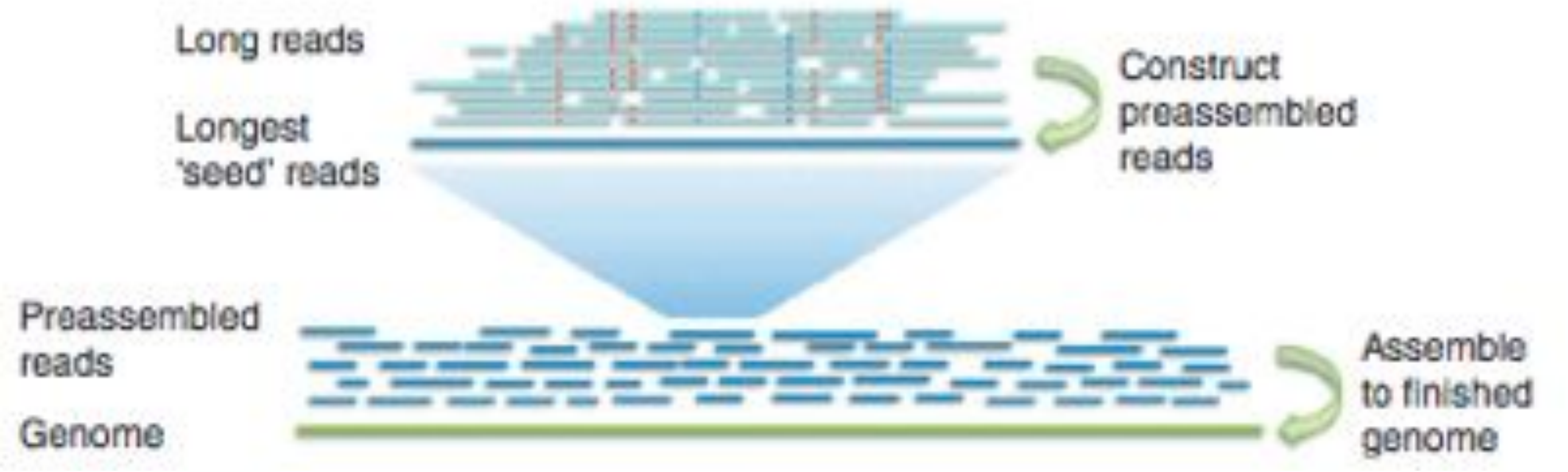


10.75x over 10kbp

Mean: 9,348

Max: 54,288bp

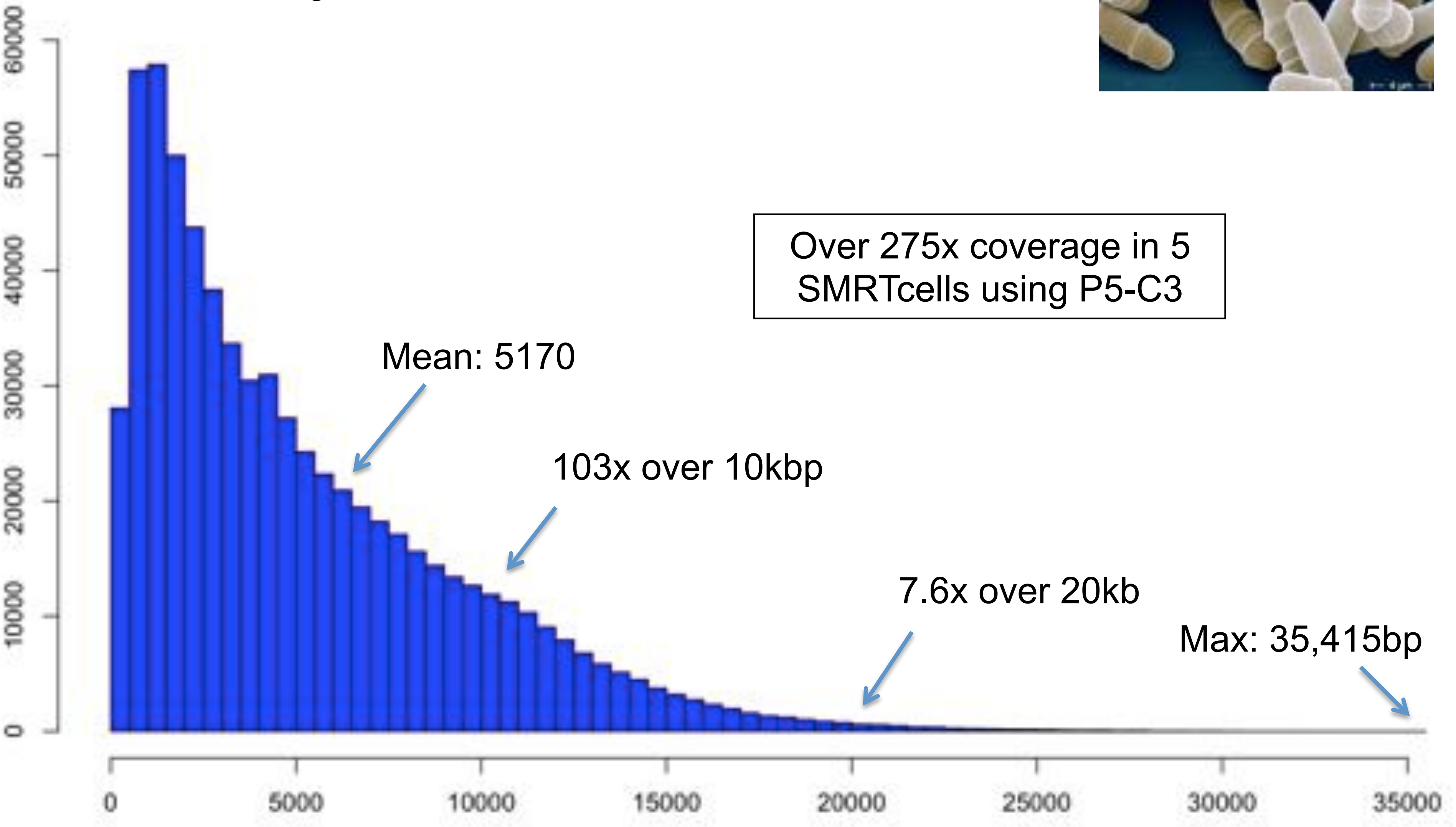
# HGAP Error Correction



# S. pombe dg21

PacBio RS II *s*equencing 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

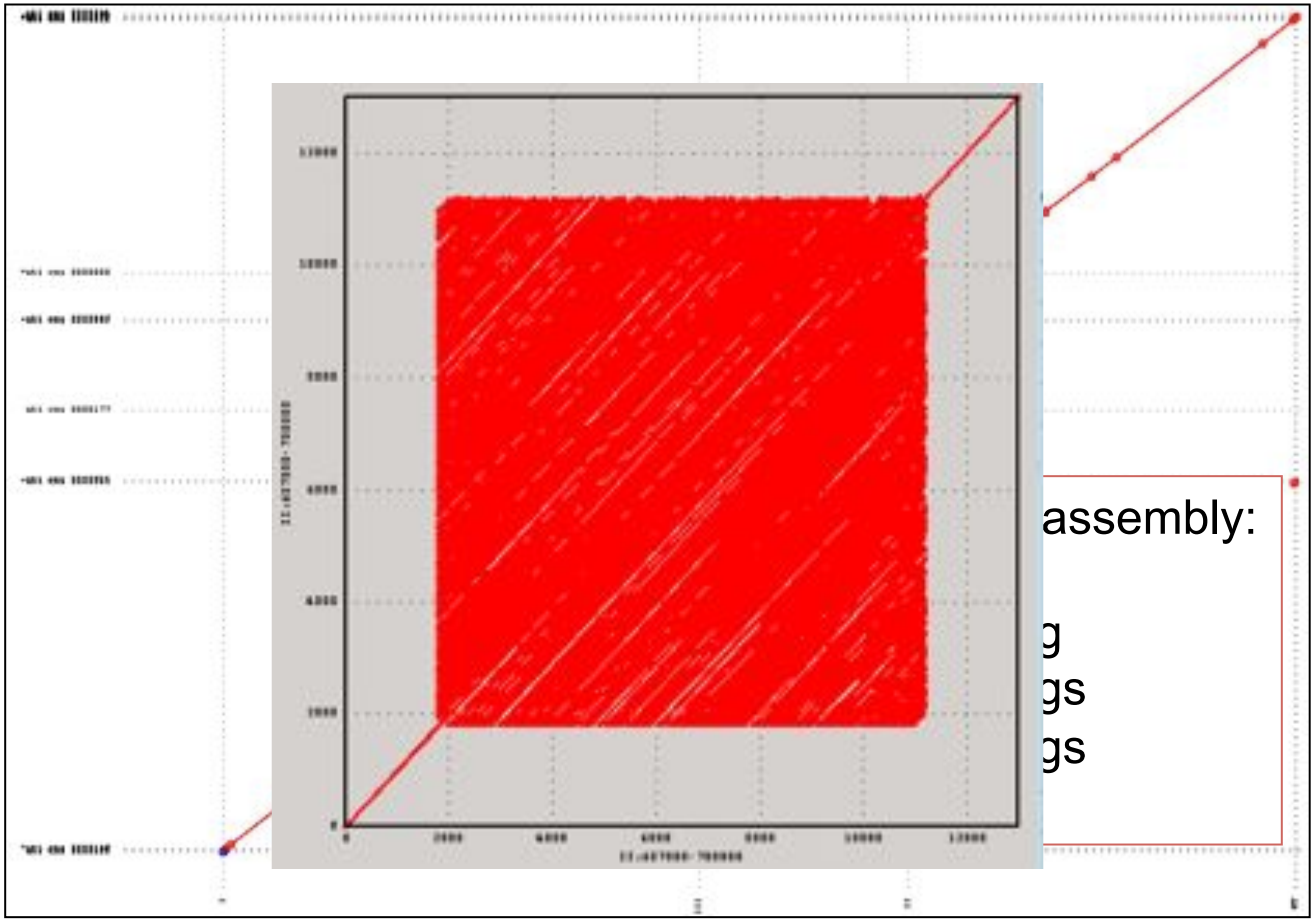
# S. pombe dg21

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

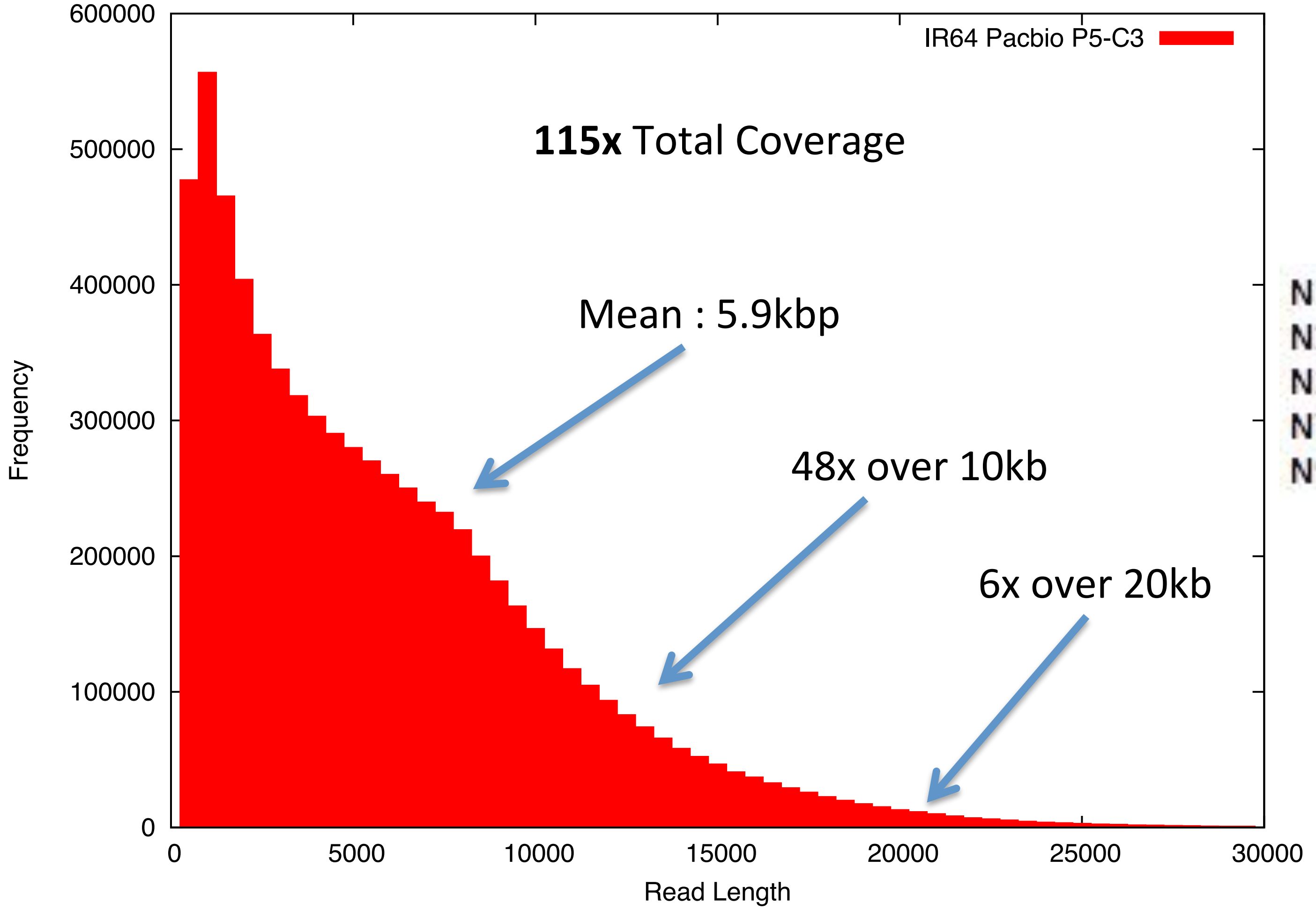


# O. sativa pv Indica (IR64)

Genome size: ~370 Mb

Chromosome N50: ~29.7 Mbp

## September 2014



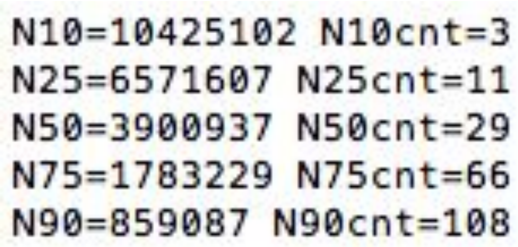
**115x** Total Coverage

Mean : 5.9kbp

48x over 10kb

6x over 20kb

Assembly Results



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

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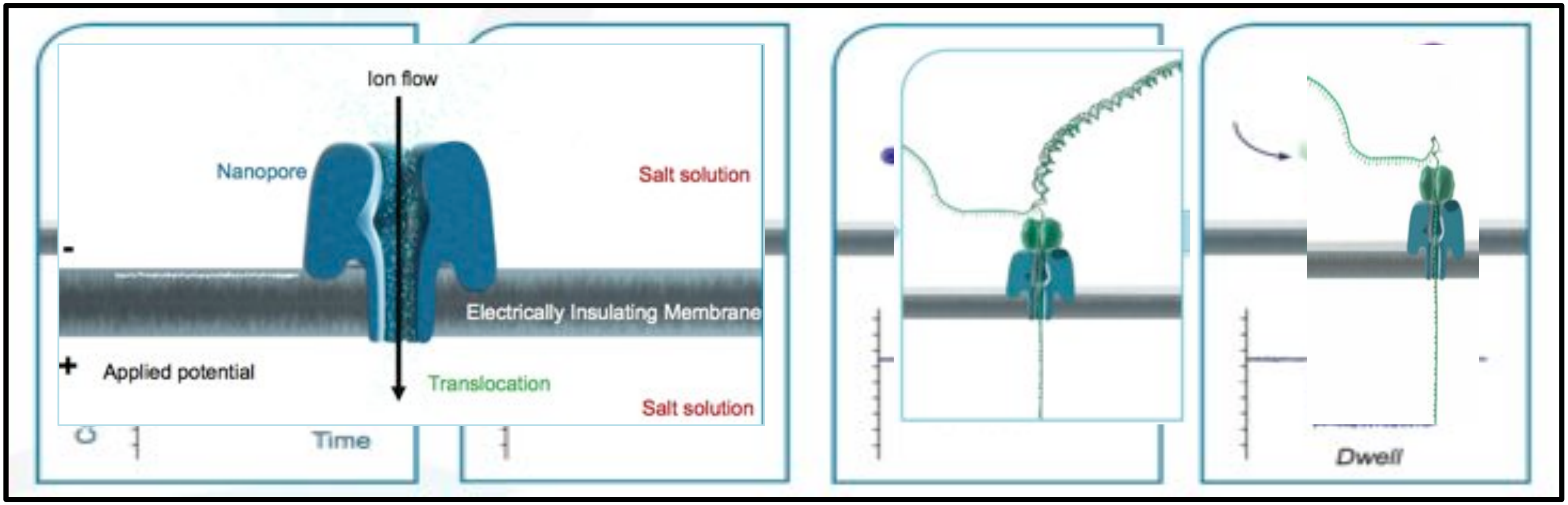
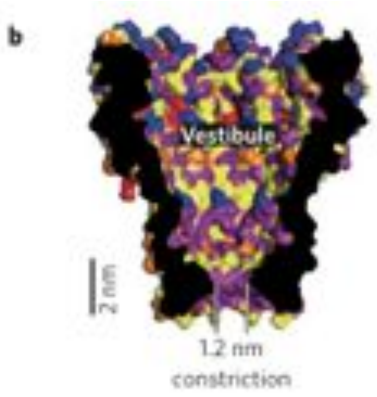
# Oxford Nanopore MinION

- 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



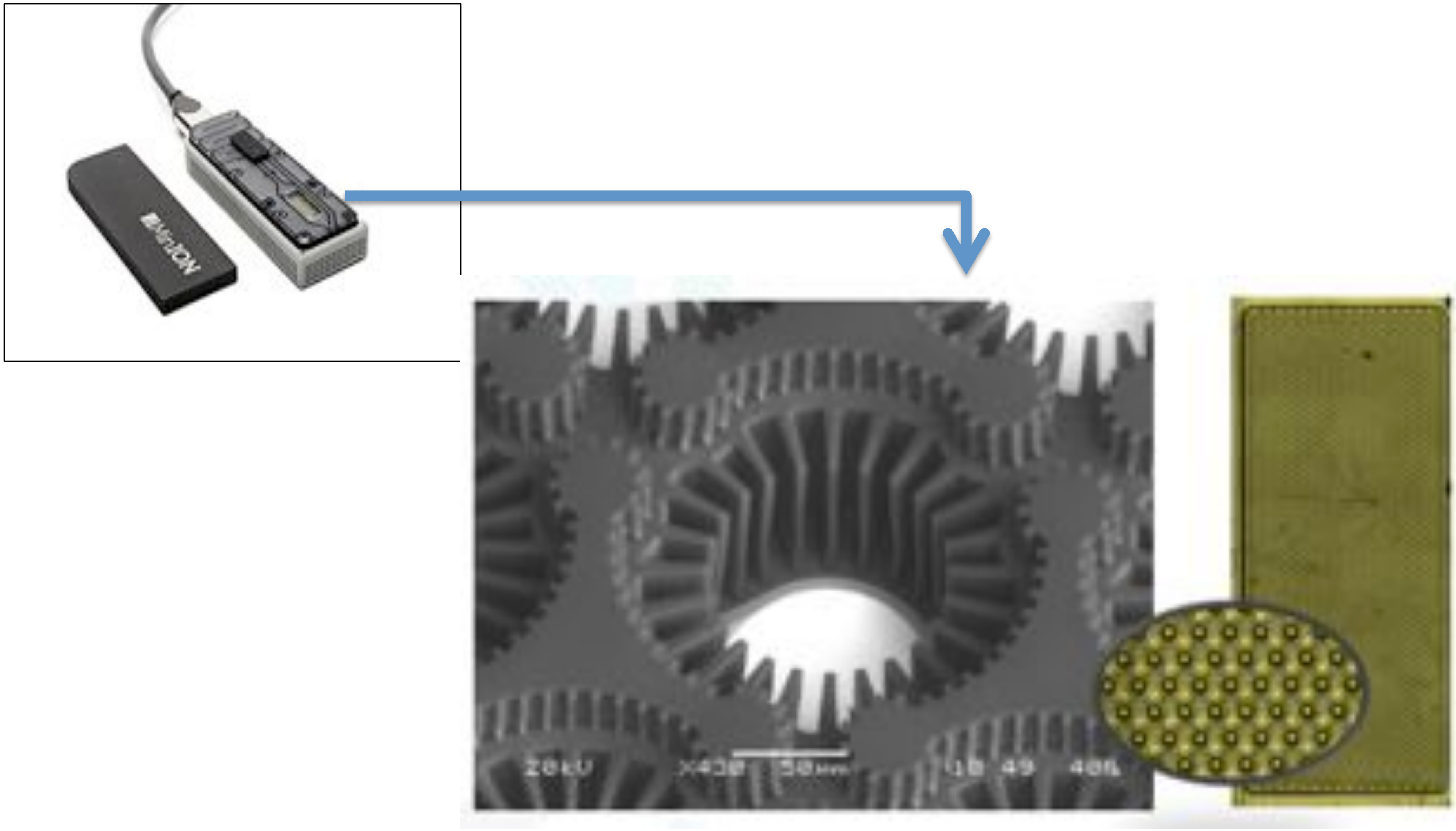
## Advantages

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

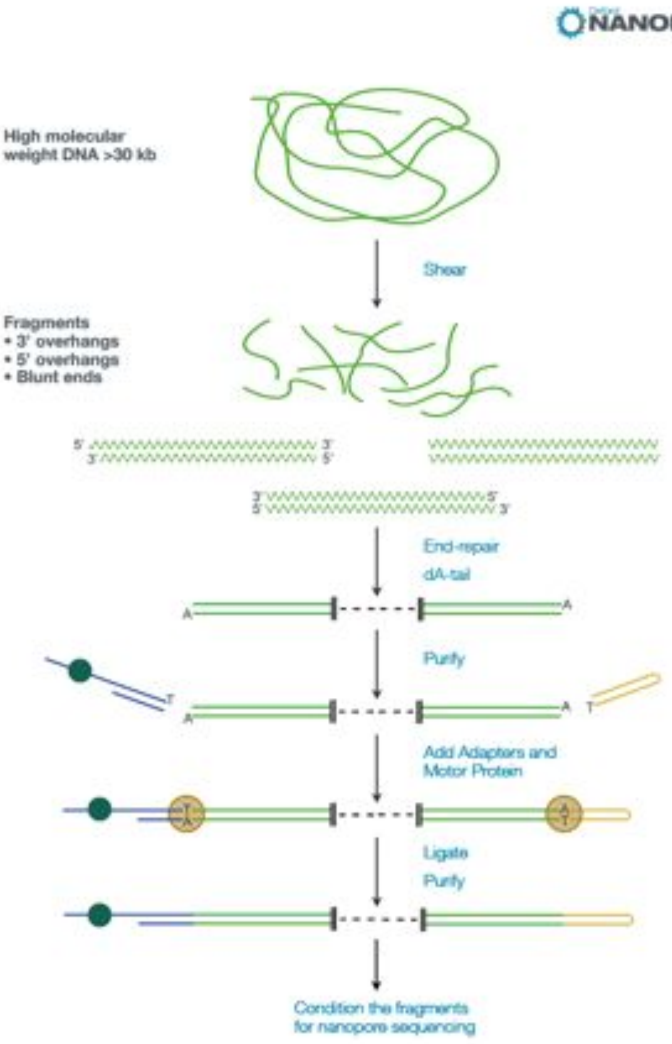
## Challenges

- 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



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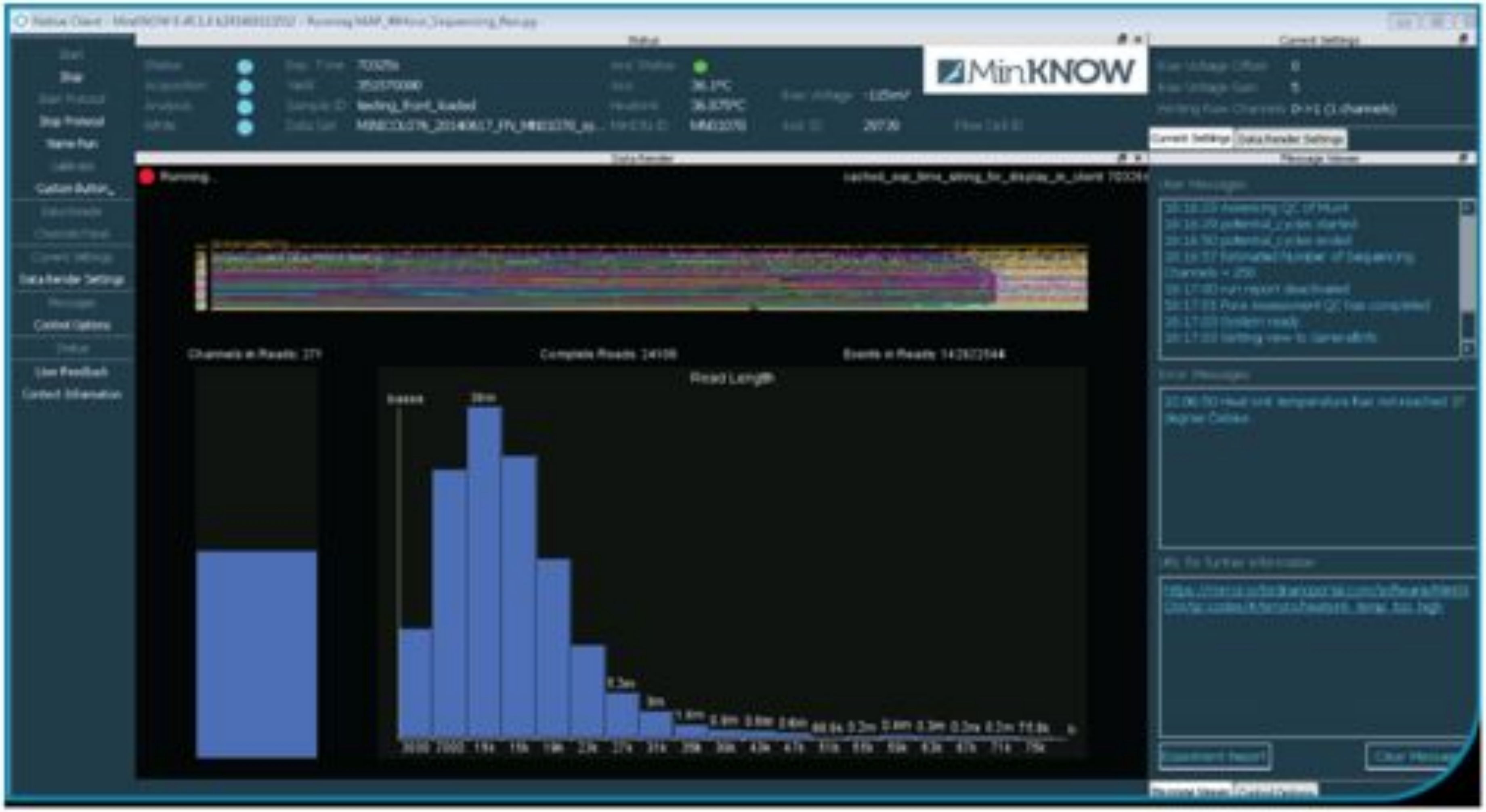
Simple DNA prep

Can do it in the field?

# Nanopore Desktop Software



Exp. Time 70325s

Yield 3515370080

Sample ID testing_front_loaded

Bias Voltage -120mV

Running...

Channels in Reads: 271

Complete Reads: 24108

Events in Reads: 142822544

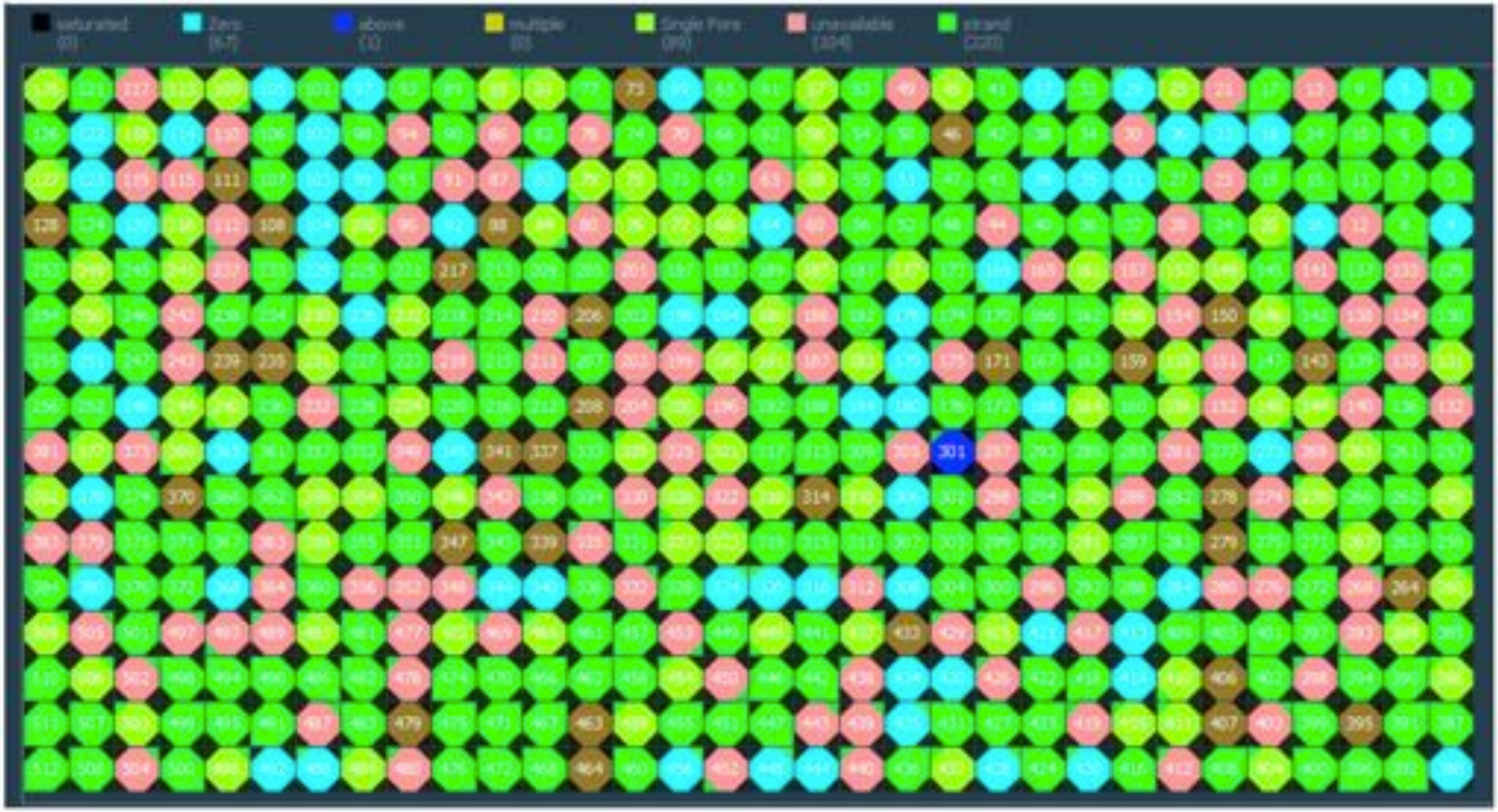
User Messages

```
16:16:33 Assessing QC of MinION
16:16:39 potential_cycles started
16:16:50 potential_cycles ended
16:16:57 Estimated Number of Sequencing Channels = 250
16:17:00 run report deactivated
16:17:01 Pore Assessment QC has completed
16:17:03 System ready
16:17:03 Setting view to General
```

Error Messages

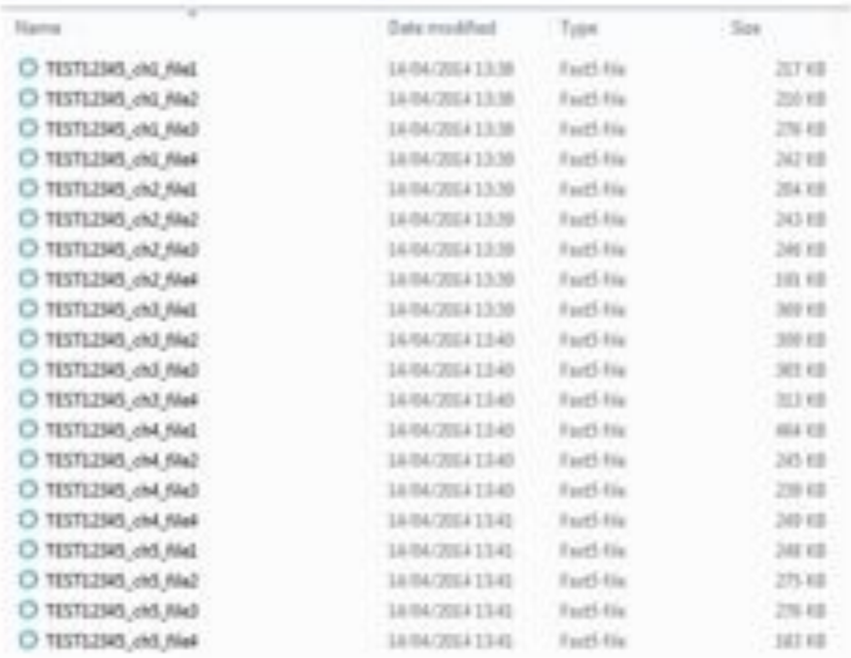
```
10:06:50 Heat sink temperature has not reached 37 degree Celsius
```

# 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 | 181 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 | 383 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

METRICHOR AGENT

Instance stopped

Uploader stopped

Downloader stopped

Log

[illegible]

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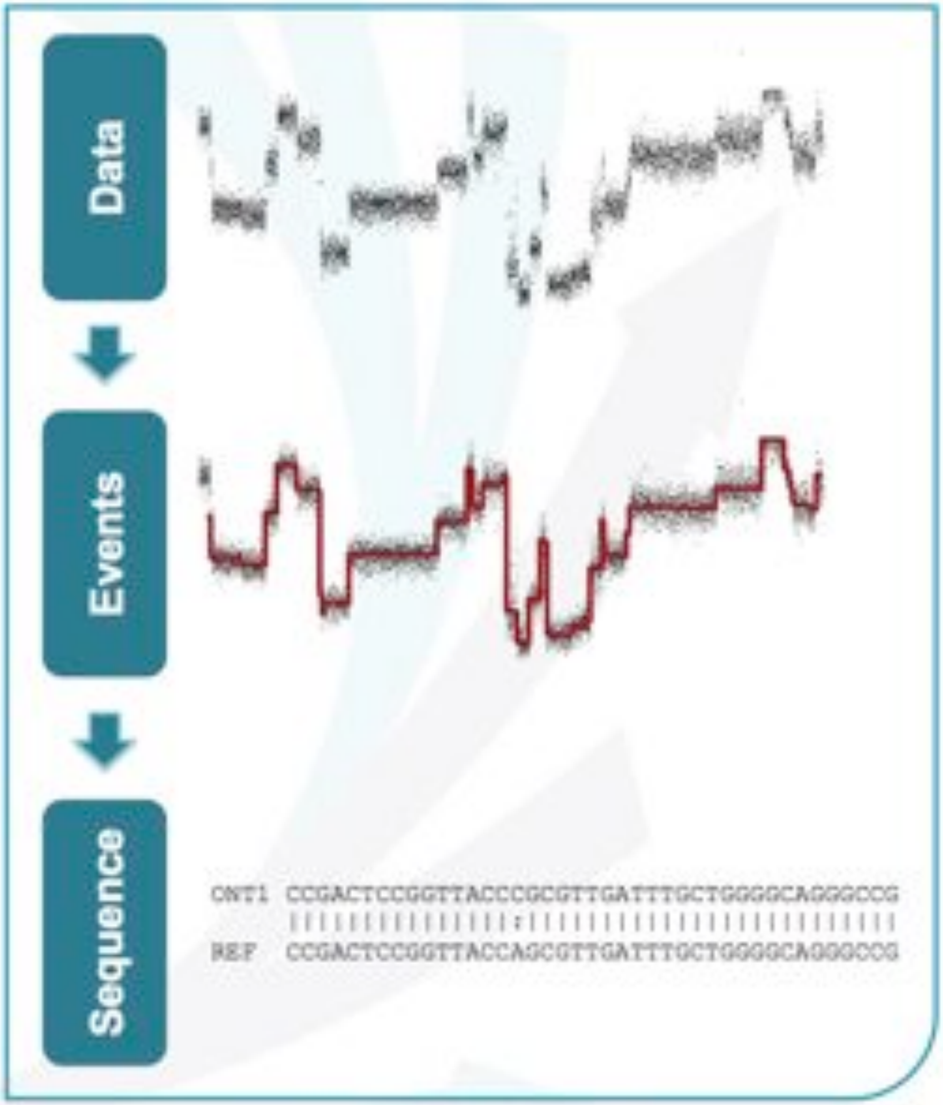
upload reads

Download fast5 with called bases

amazon web services

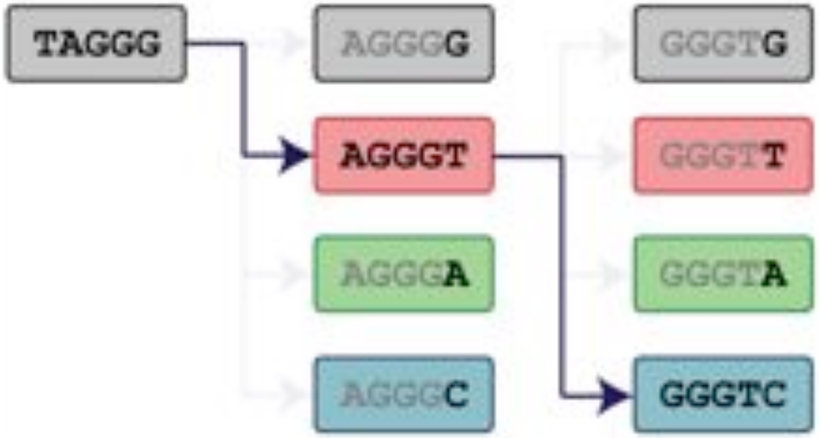
Base Calling in Cloud

# Nanopore Basecalling



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

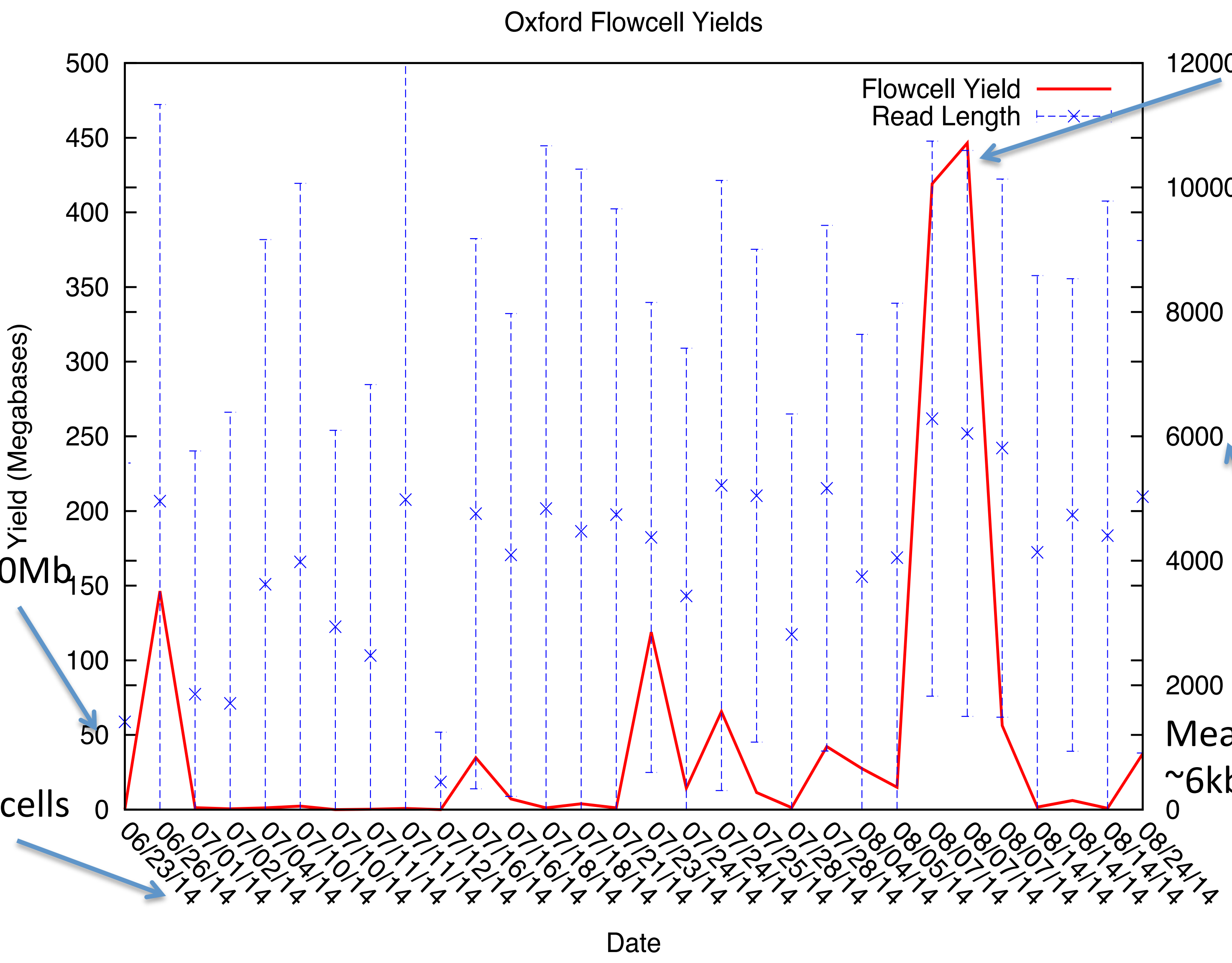
- Hidden Markov model
- Only four options per transition
- Pore type = distinct kmer length



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

Basecalling currently performed at Amazon with frequent updates to algorithm

# Our Data - Yeast W303



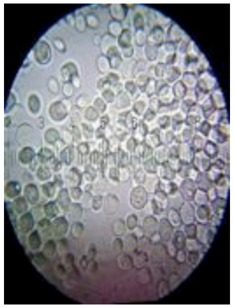
Best: 446Mb

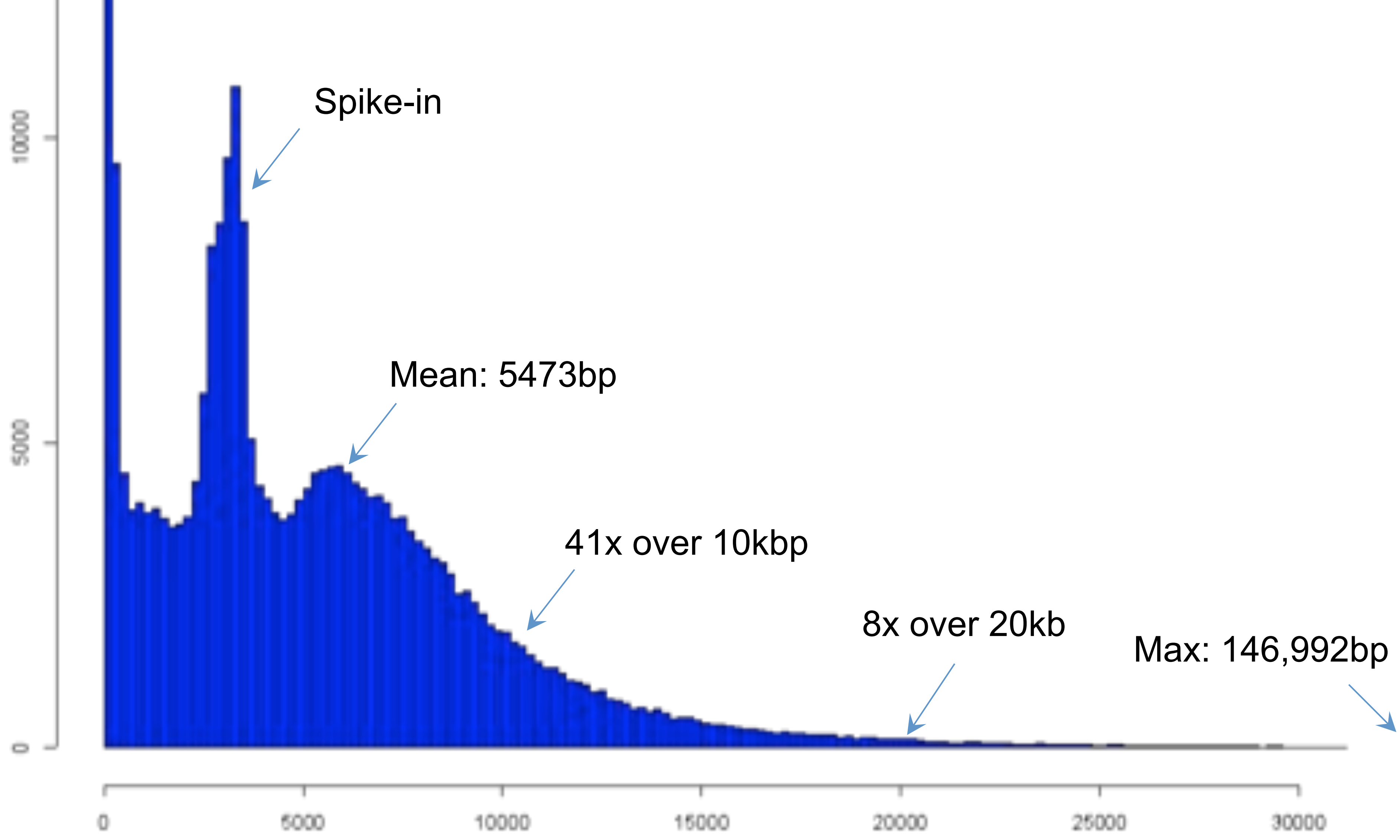
Mean: 50Mb

30 Flowcells

Mean Read Length ~6kb (10kb shear)

# Nanopore Readlengths





noise

Spike-in

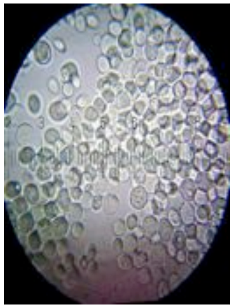
Mean: 5473bp

41x over 10kbp

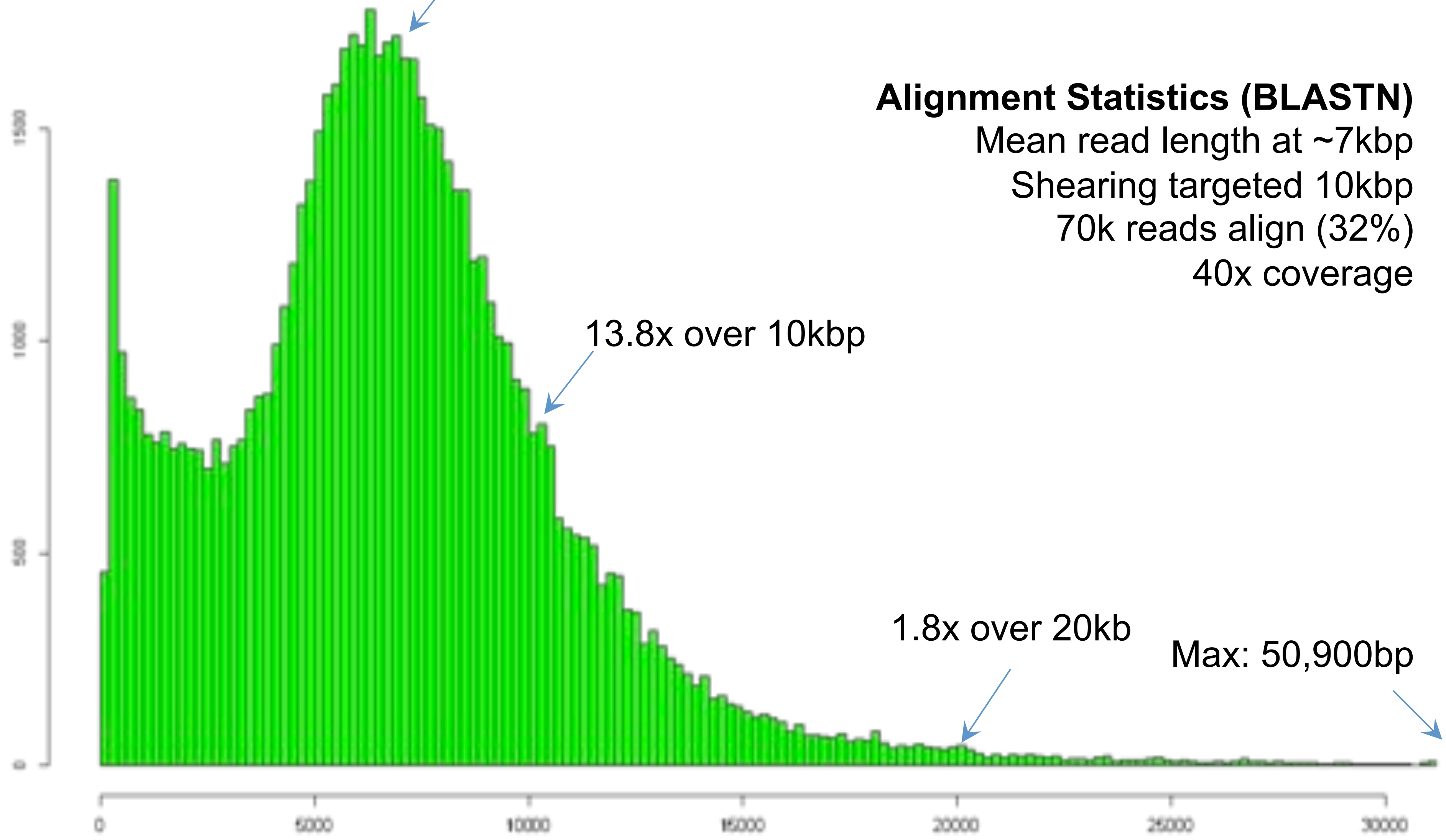
8x over 20kb

Max: 146,992bp

# Nanopore Alignments



**Alignment Statistics (BLASTN)**
Mean read length at ~7kbp
Shearing targeted 10kbp
70k reads align (32%)
40x coverage



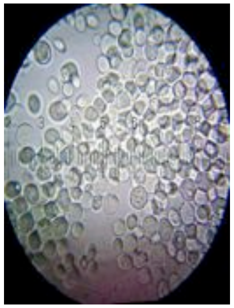
Mean: 6903bp

13.8x over 10kbp

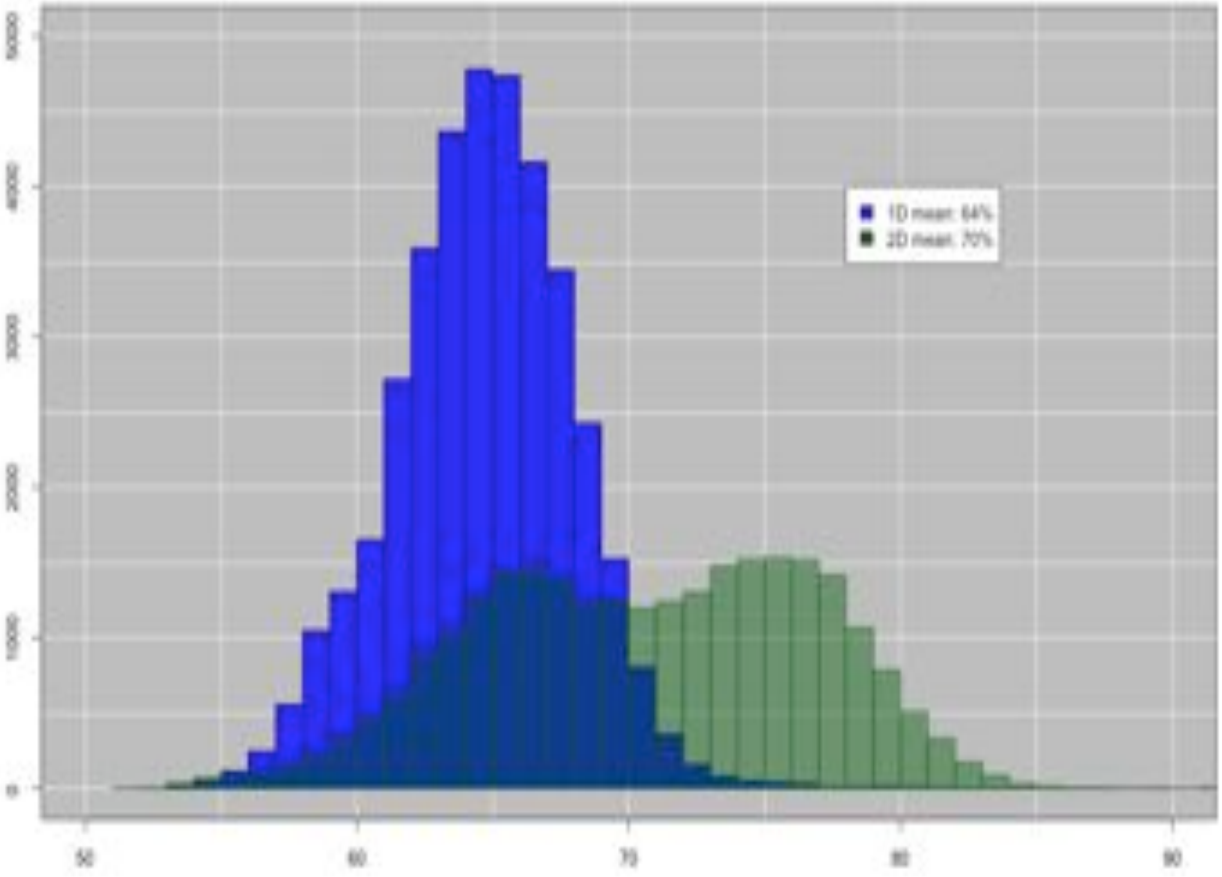
1.8x over 20kb

Max: 50,900bp

# Nanopore Accuracy



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

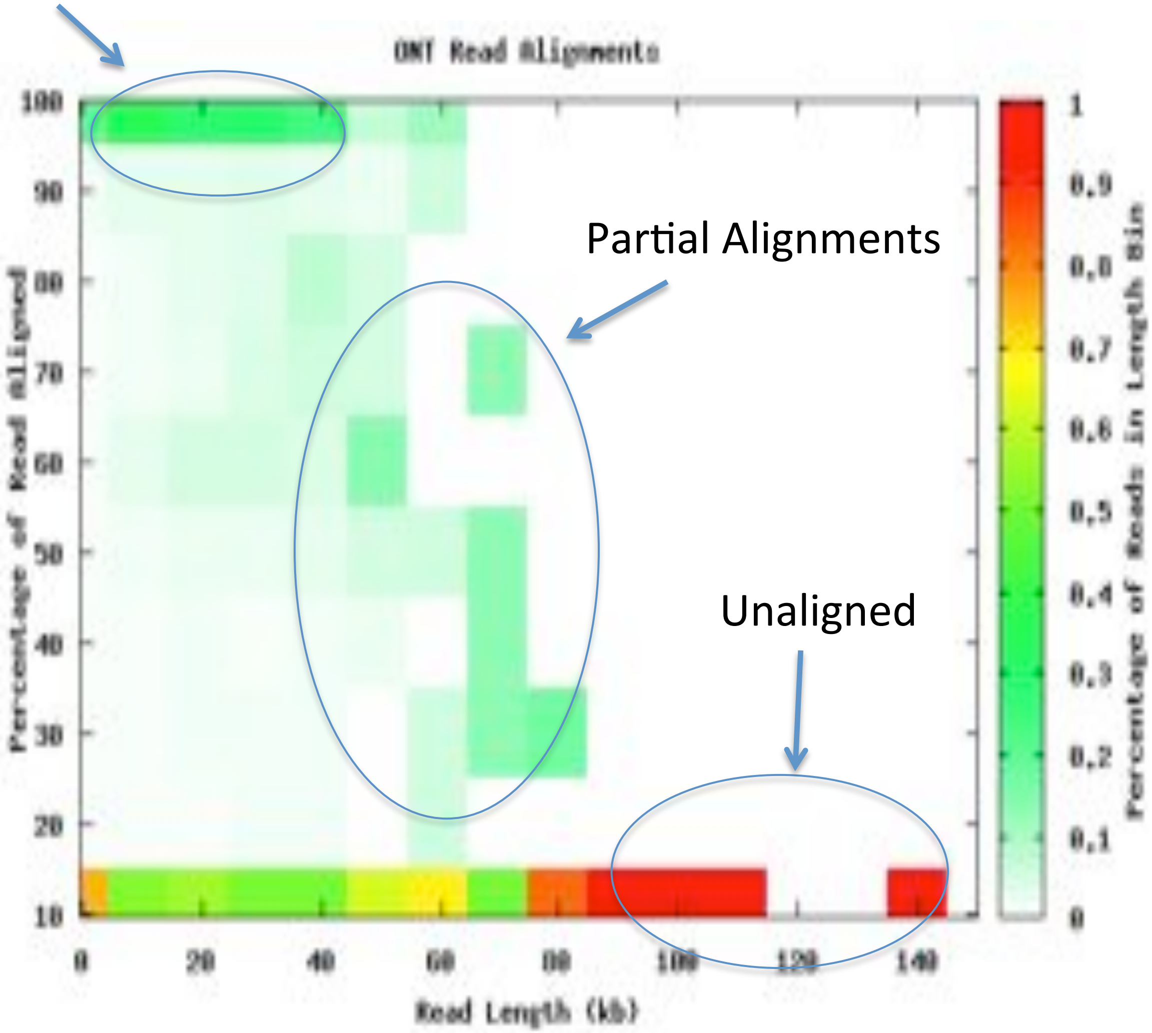


57% Mismatches
32% Deletions
11% Insertions

# Nanopore Alignment Summary

32% of the data map using BLASTN

Full Length Alignments



Partial Alignments

Unaligned

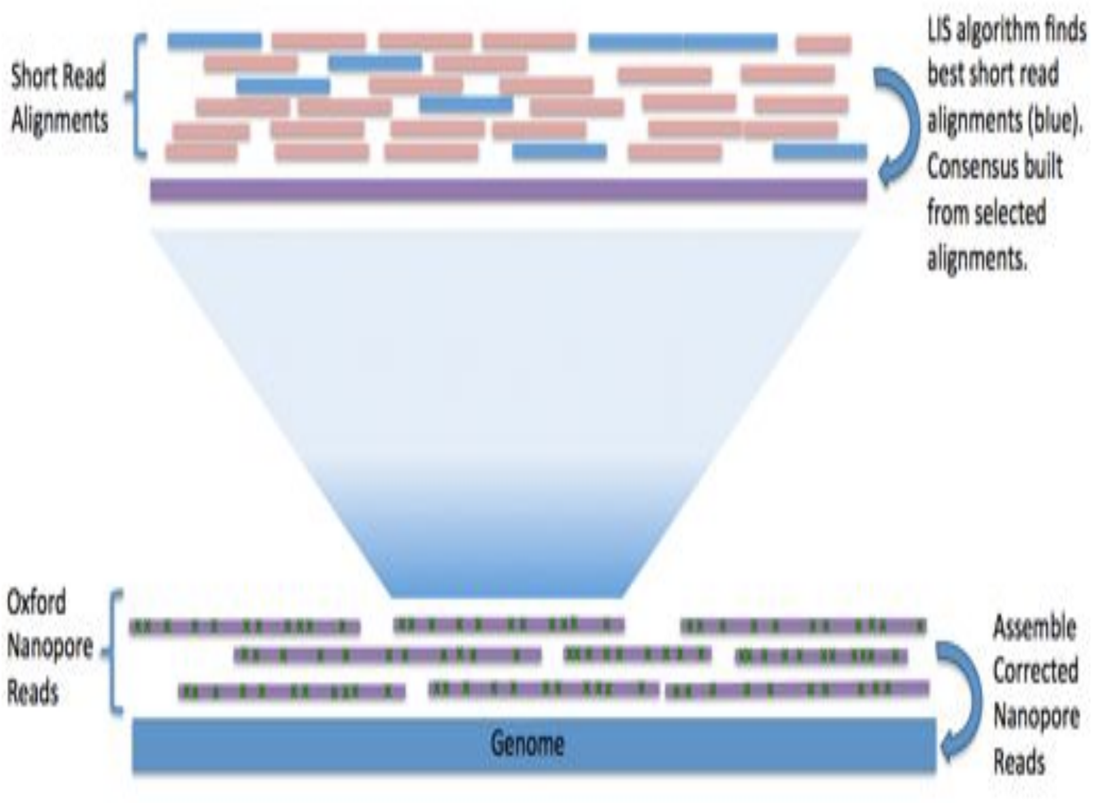
# NanoCorr: Nanopore-Illumina Hybrid Error Correction

https://github.com/jgurtowski/nanocorr

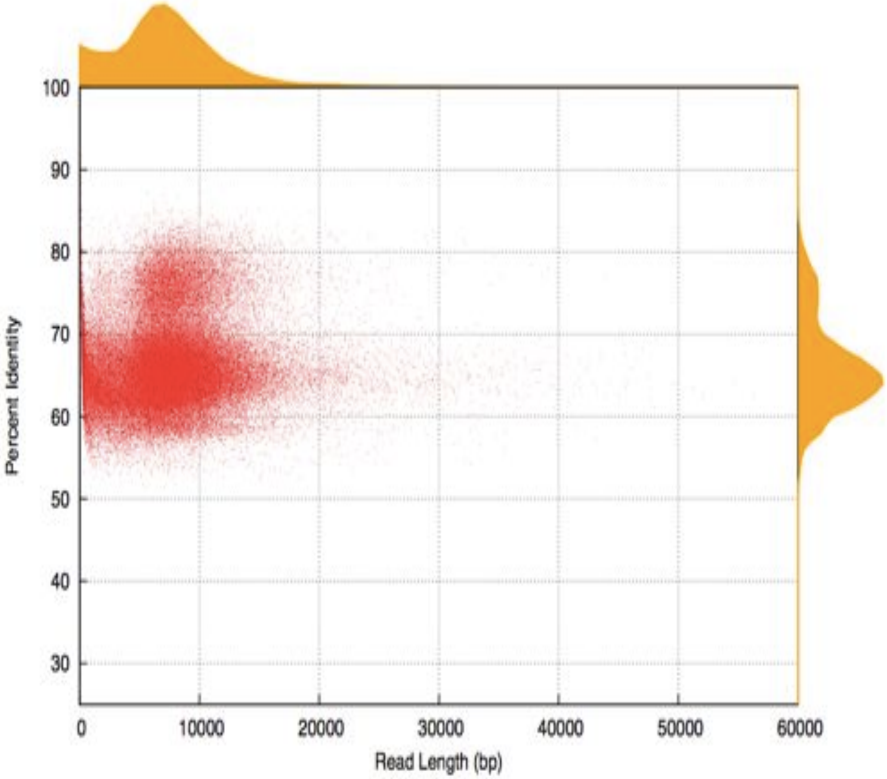
1. BLAST Miseq reads to all raw Oxford Nanopore reads

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

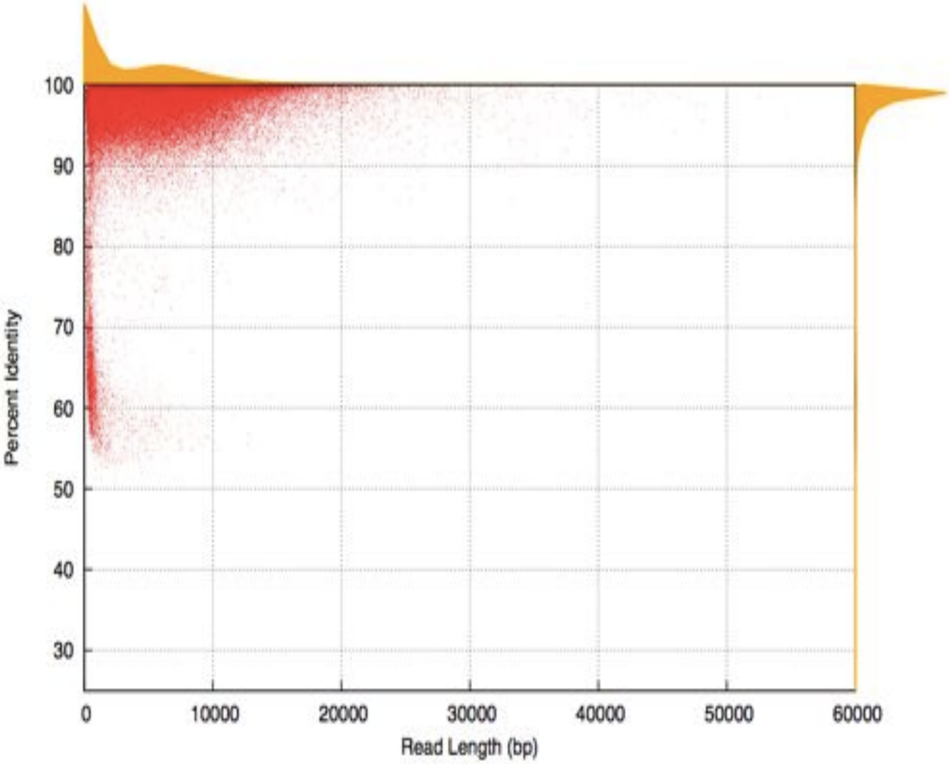
3. Compute consensus of each Oxford Nanopore read
   1. Currently using Pacbio's pbdagcon


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

# Nanocorr correction pipeline significantly improves read identity



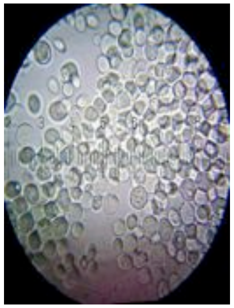
Before



After

Percent identity versus read length before and after nanocorr correction

# Long Read Assembly

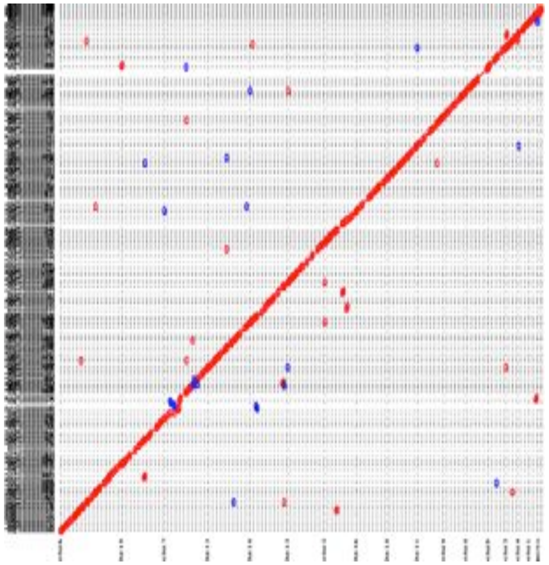


## S288C Reference sequence

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

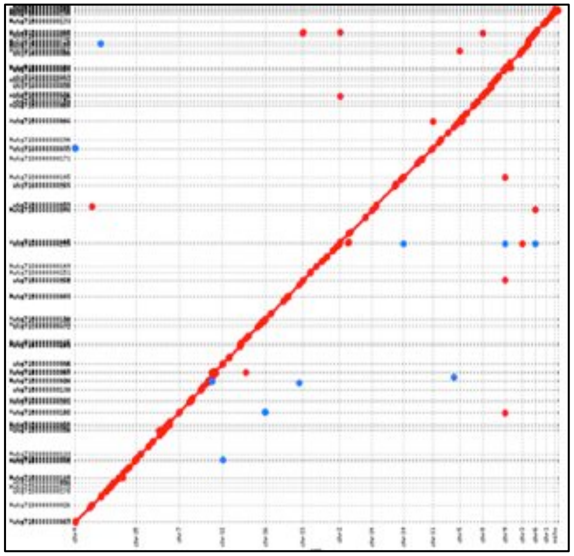
***Illumina MiSeq***
30x, 300bp PE (Flashed)
Celera Assembler

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



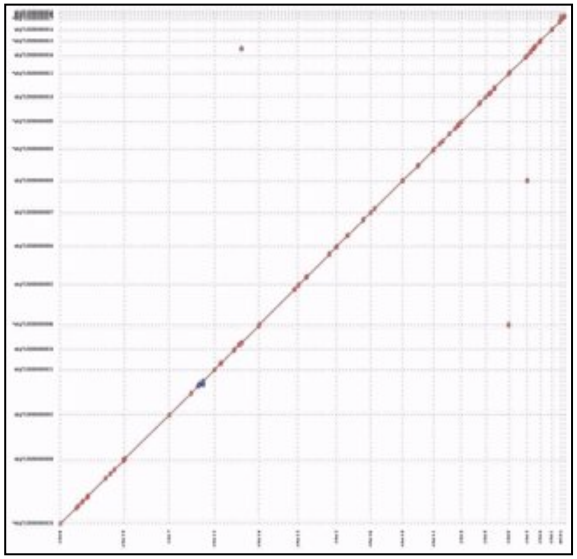
***Oxford Nanopore***
30x corrected reads > 6kb
NanoCorr + Celera Assembler

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

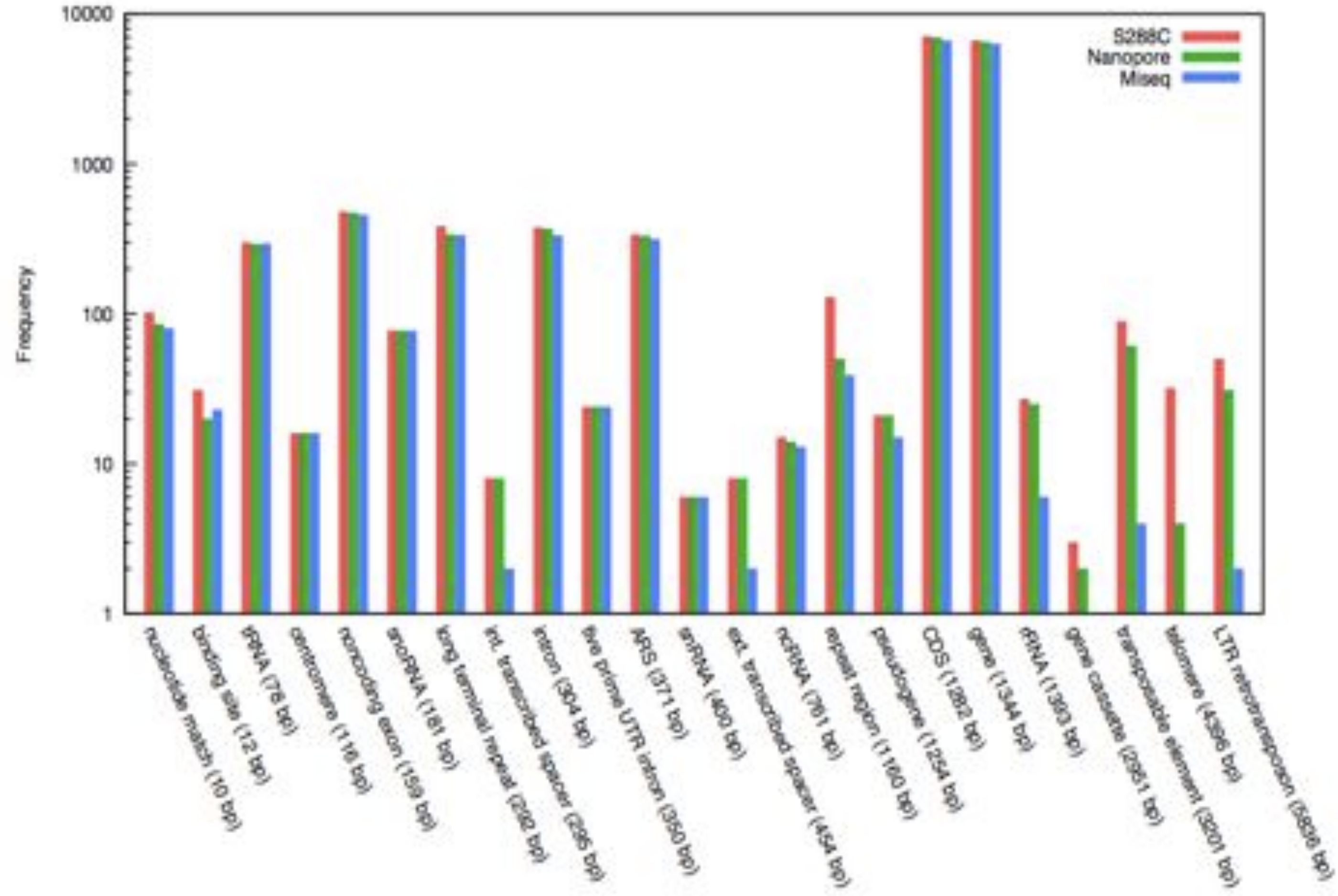


***Pacific Biosciences***
25x corrected reads > 10kb
HGAP + Celera Assembler

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



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

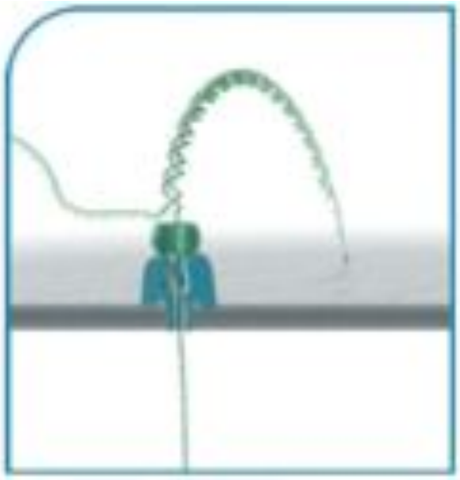


- S288C is an extremely high quality reference

- 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

# Summary

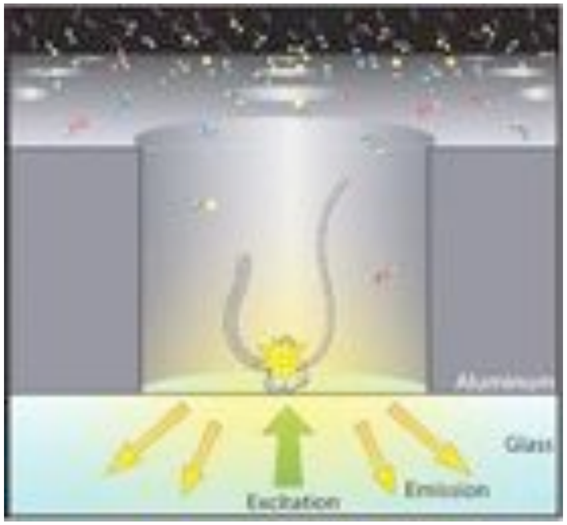


1. New Single Molecule Sequencing Technologies

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



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