Genomic Medicine Short Course
Kansas City, MO
September 2019

JOHNS HOPKINS
BIOMEDICAL ENGINEERING

Answering questions with nanopore sequencing: From bacteria to sequoias

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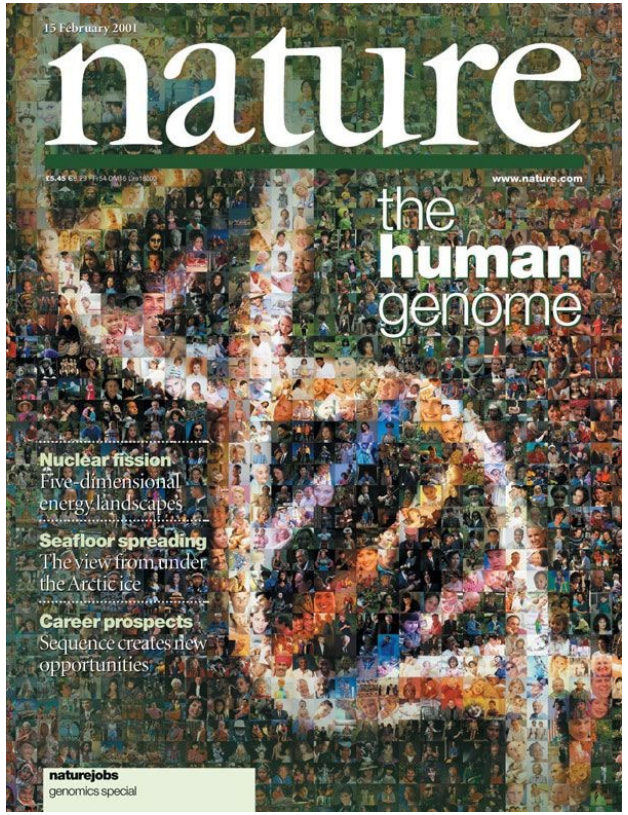
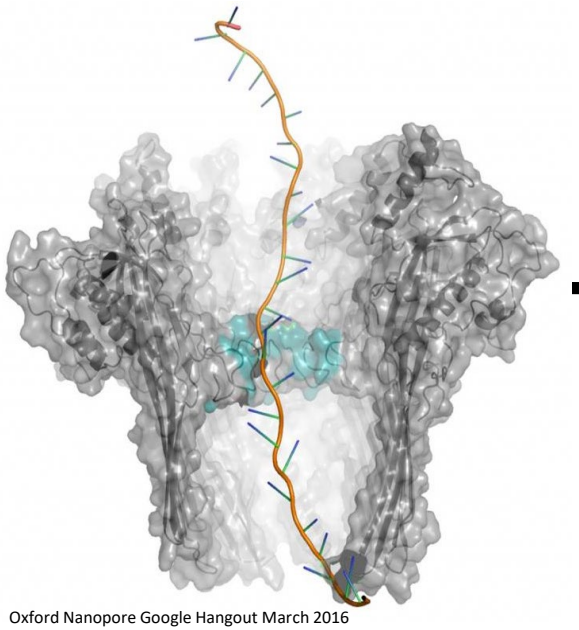
Revolutions in Science: Genomics

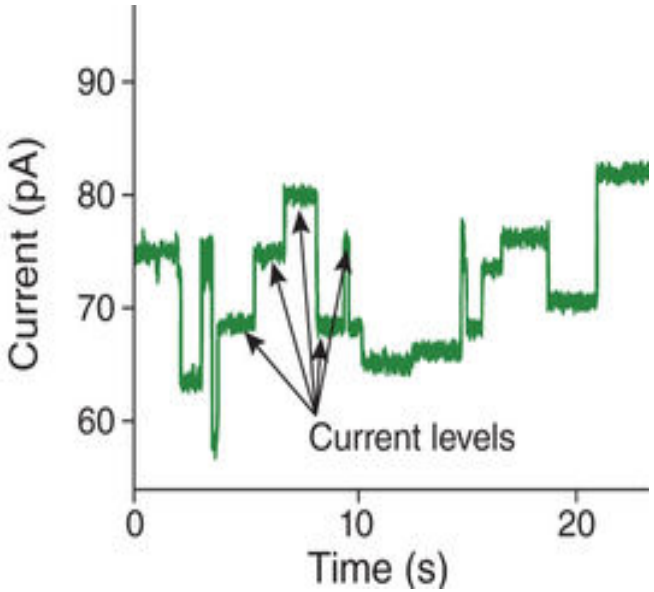


- Draft of the human genome was completed in 2001
- ~3 billion bases in size
- Think about this like the first transistor (1947) – the watershed after which genomic and epigenomic engineering has exploded

Revolutions in Genomics: Single Molecule Sequencing

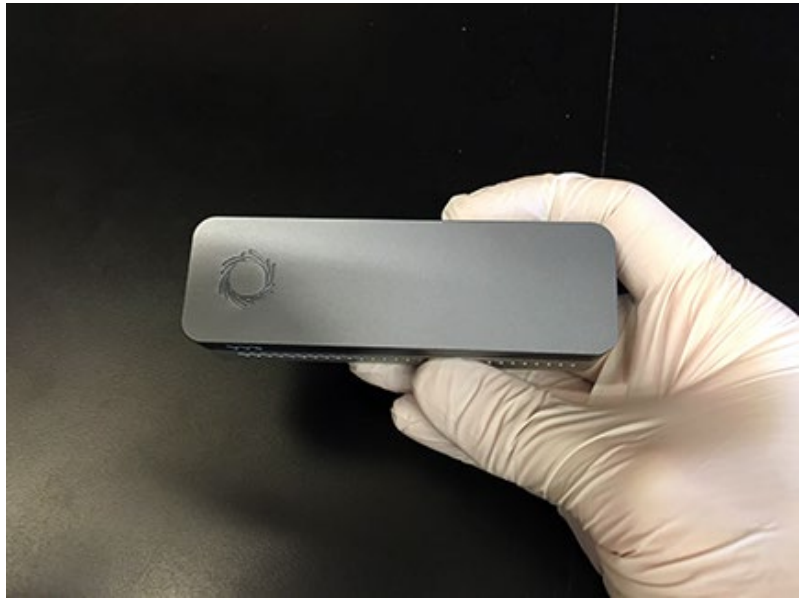


Oxford Nanopore Google Hangout March 2016



Deamer et al 2016, Nature Biotech

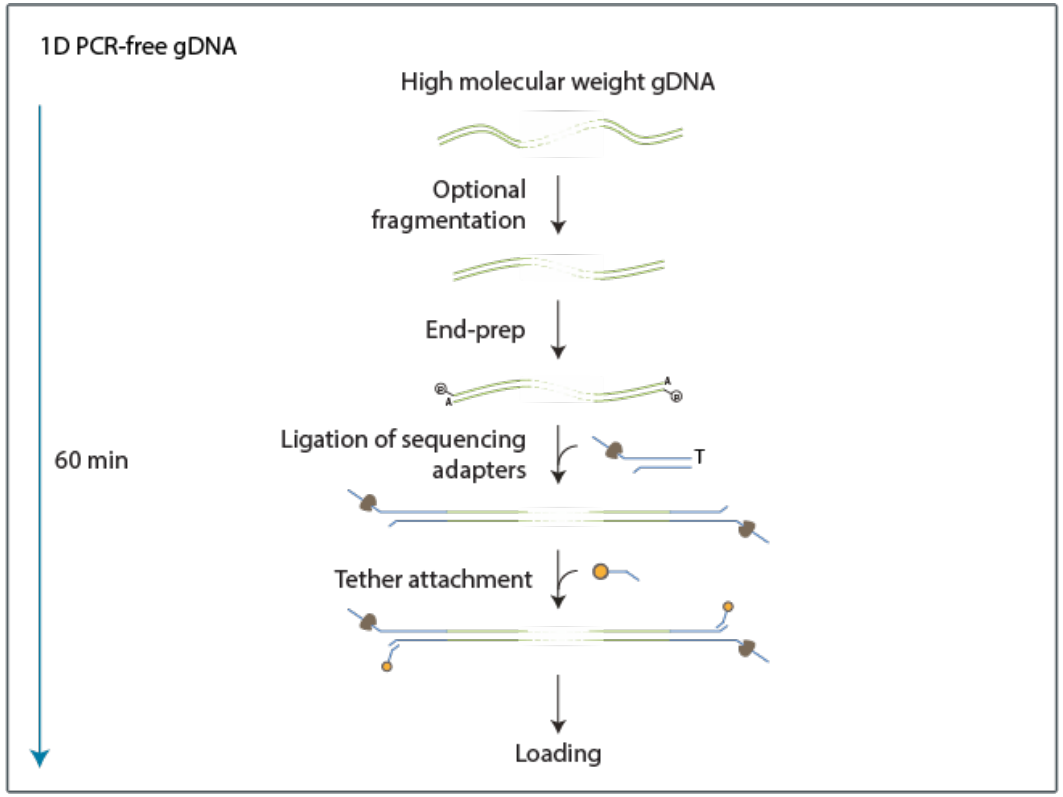
ATCGATCGATAGTA
TTAGATACGACTAG
CGATCAG

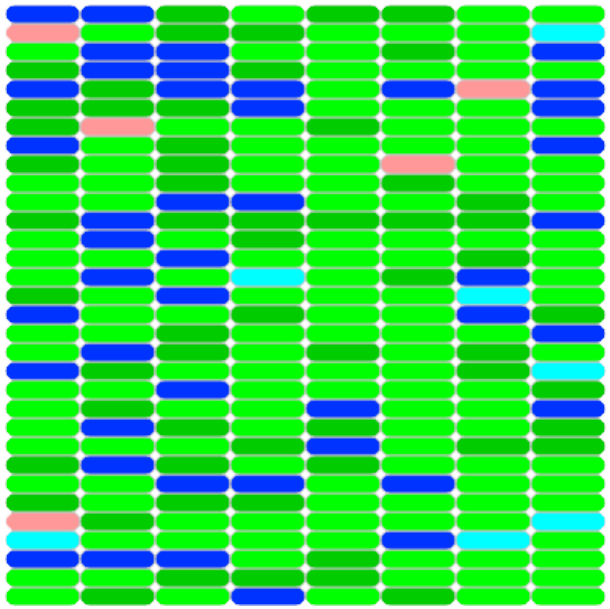


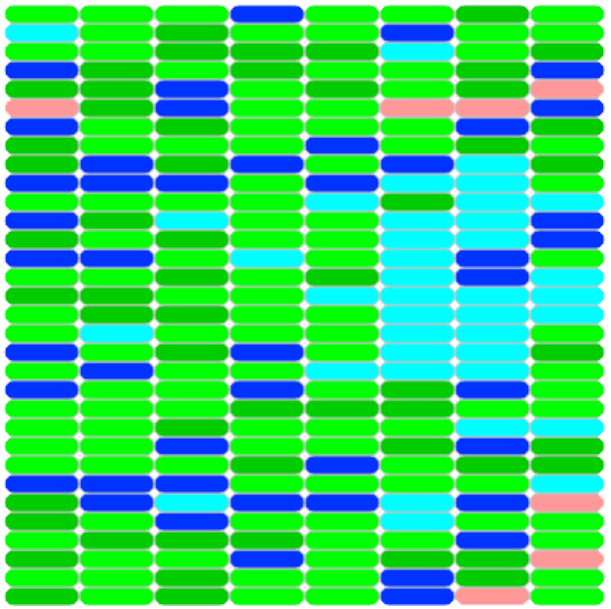
- First patented back in 1995, commercialized in 2014
- No theoretical upper limit to sequencing read length, practical limit only in delivering DNA to the pore intact
- Palm sized sequencer
- Sequencing output 5-20Gb

Disclosure: Timp has two patents (US Patent 8,748,091; US Patent 8,394,584) licensed to ONT

Nanopore Library Prep

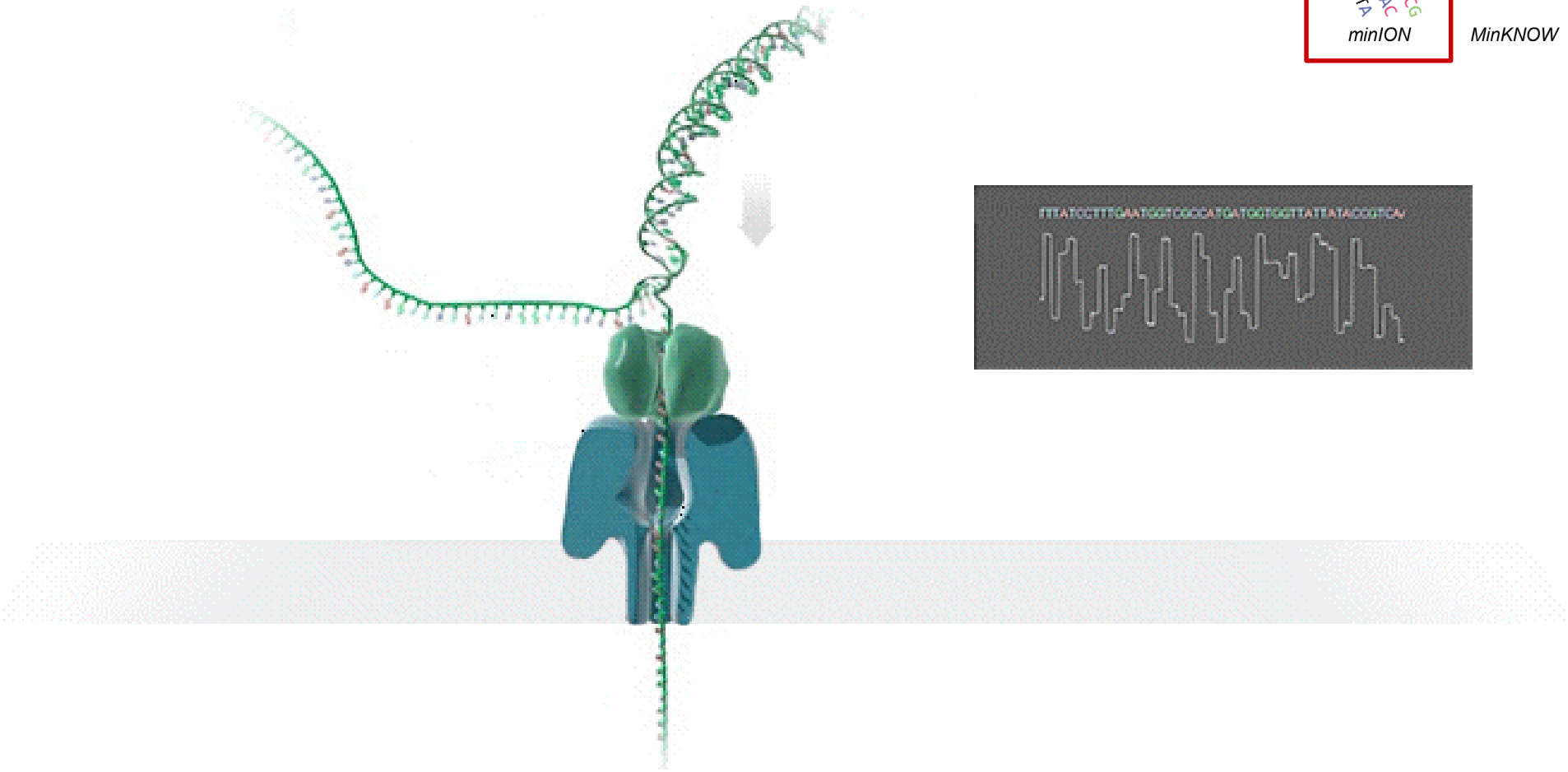


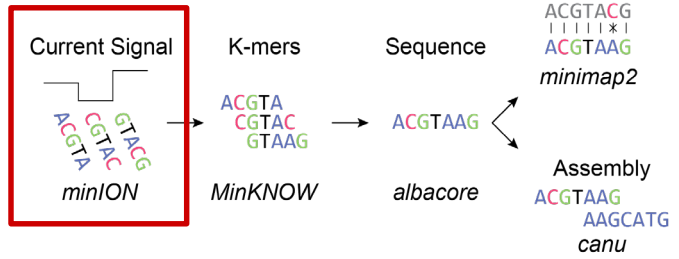




- Library prep is very similar to methods for short-read sequencing
- For DNA shearing we used Covaris gTubes or Diagenode Megaruptor
- After end-repair and A-tailing, leader adapter with motor protein is ligated
- MinION arrays 512 channels (with 4 pores possible per channel) (shown bottom left from running software); dark green pores are sequencing, light green available, other colors inactive.

Sequencing Operation

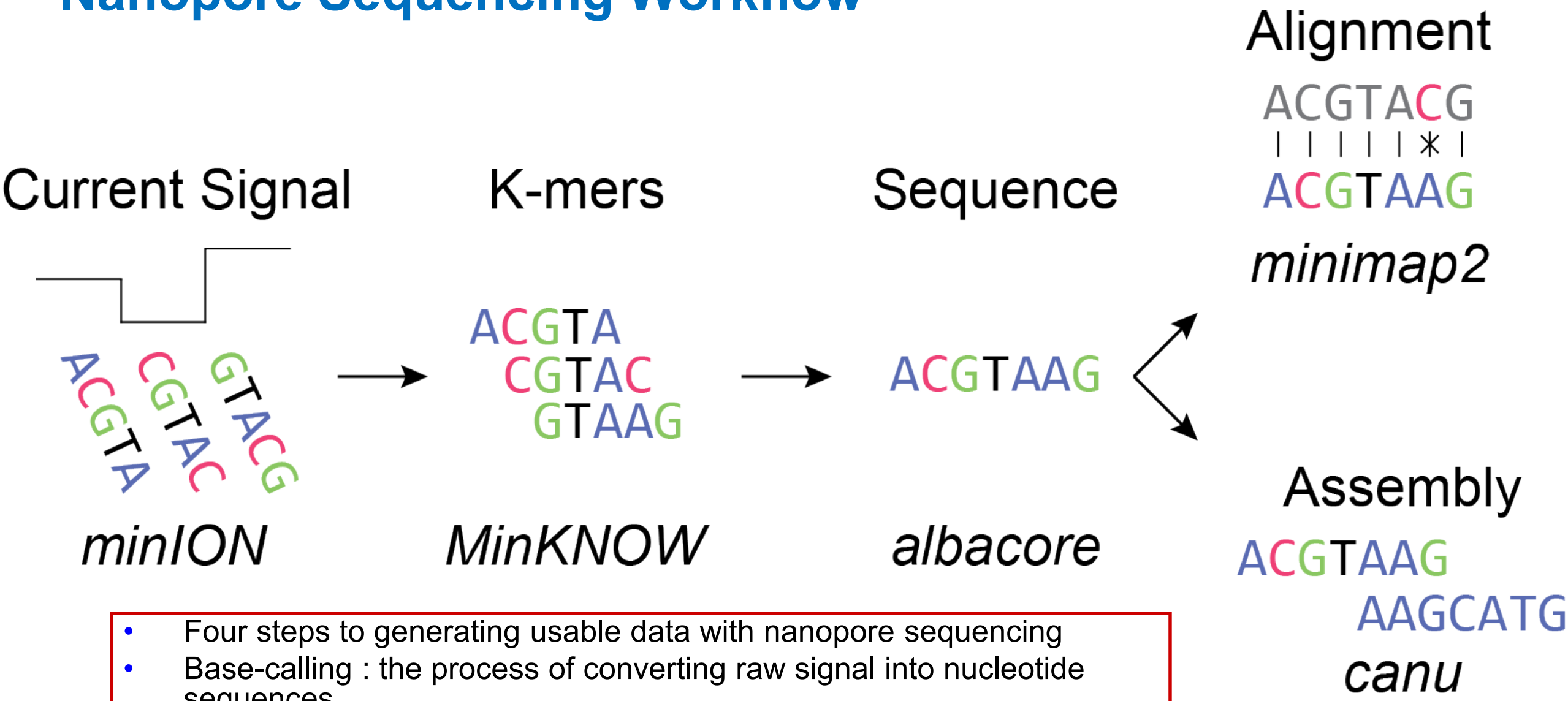




Oxford Nanopore Technologies

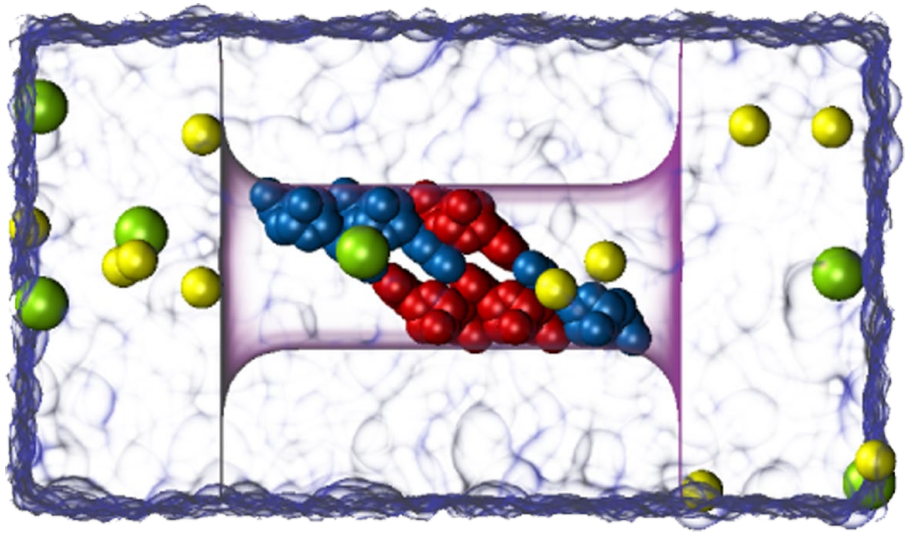
- Protein nanopores on a synthetic polymer
- Multiple base-pairs at a time (“k-mers”)
- Characteristic current signature is converted to nucleotide sequences

Nanopore Sequencing Workflow

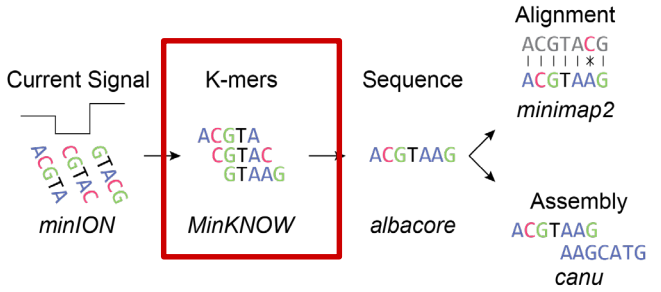


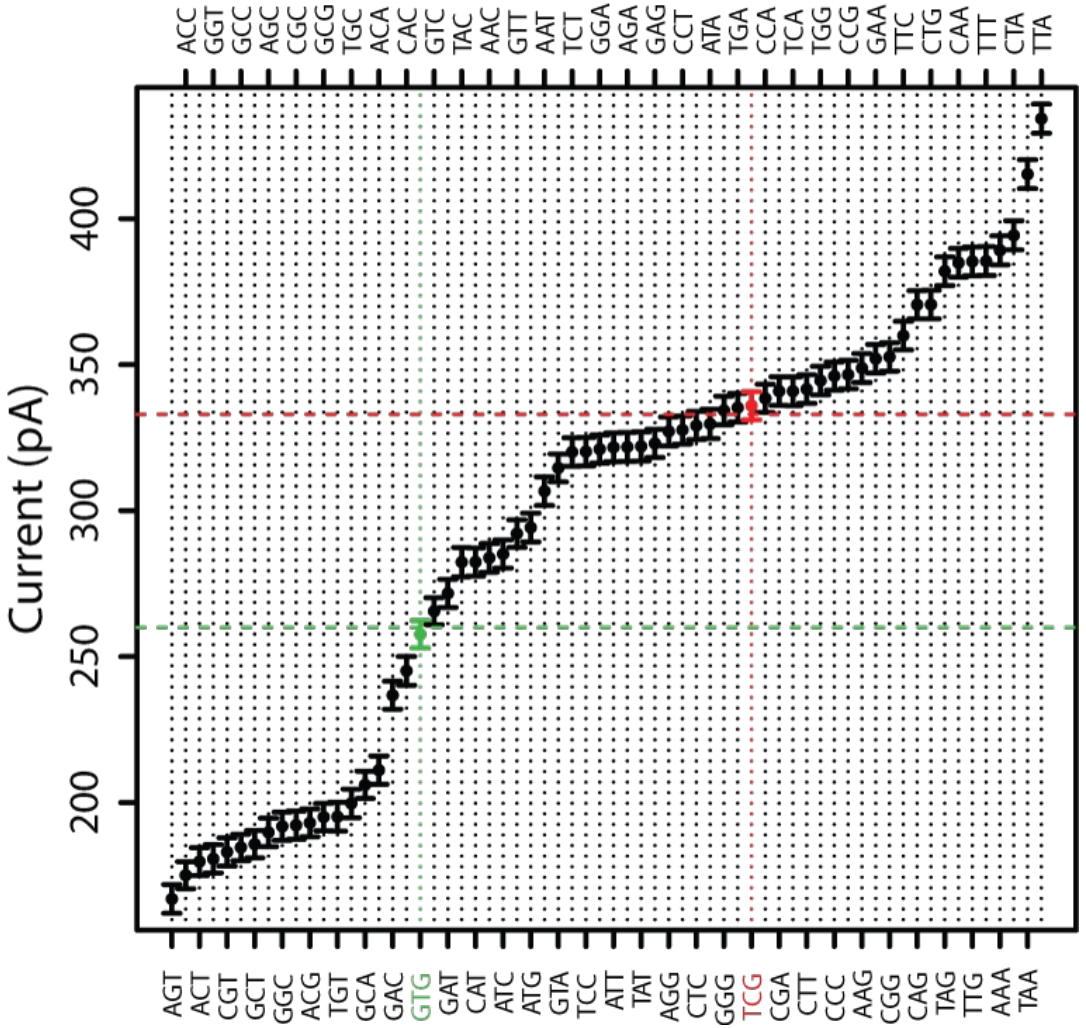
- Four steps to generating usable data with nanopore sequencing
- Base-calling : the process of converting raw signal into nucleotide sequences
- Nanopolish : uses alignment and current signal to improve base-calls

Problems with Nanopore basecalling



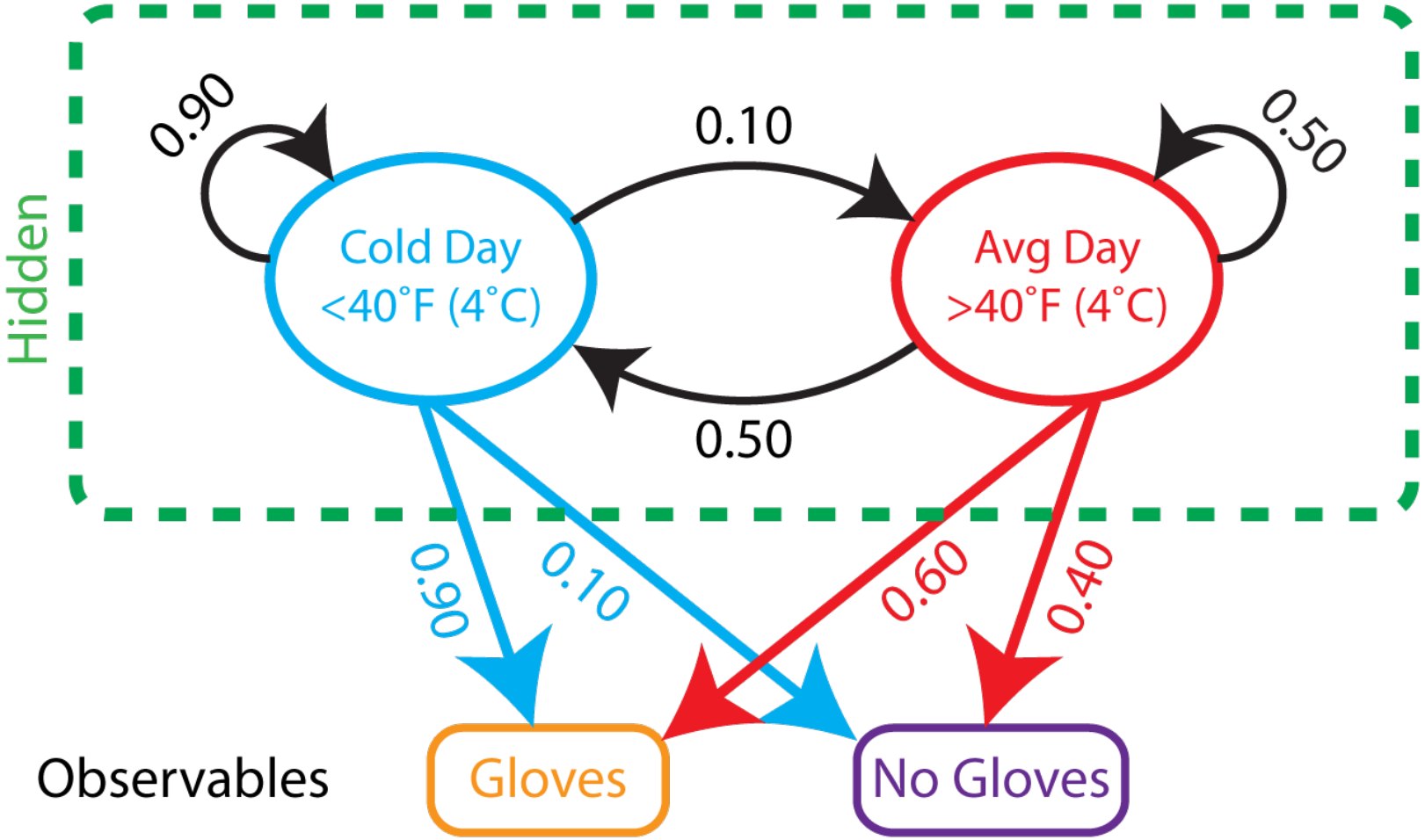
- Multiple bases influence the current passing through the pore.
- Through simulation with Brownian Dynamics, we calculated the contribution from triplets of DNA in a solid-state nanopore - 64 current levels.
- Not all of these different currents are distinguishable





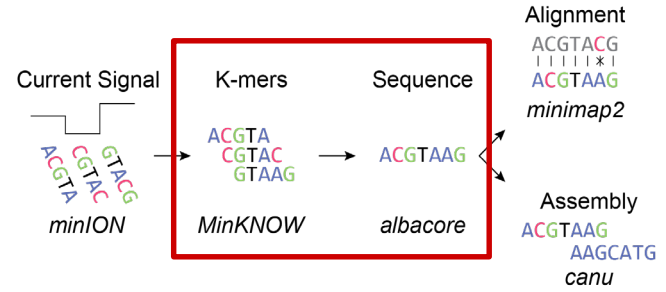
Comer and Aksimentiev *J. of Phys Chem C* 116(5) 3376-3393 (2012)

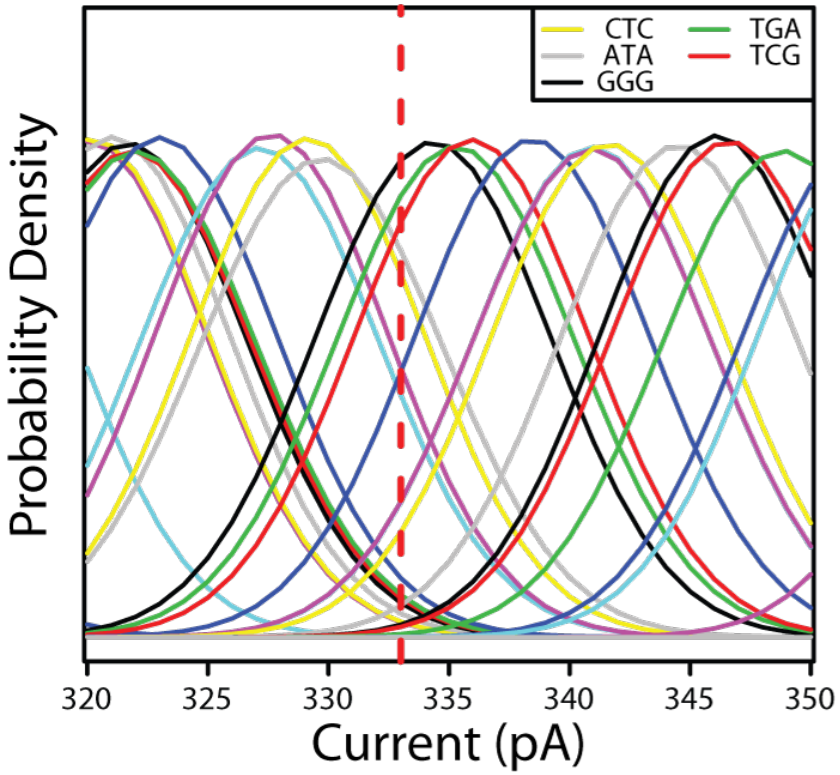
Hidden Markov Model

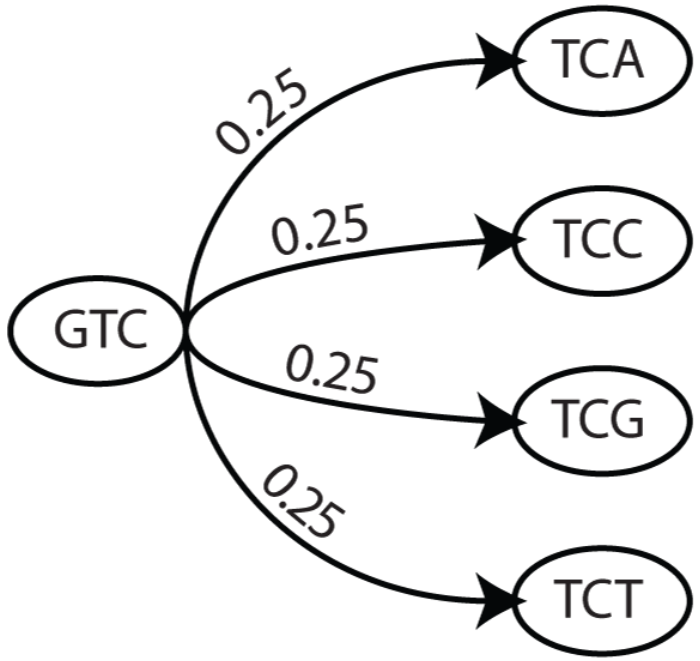


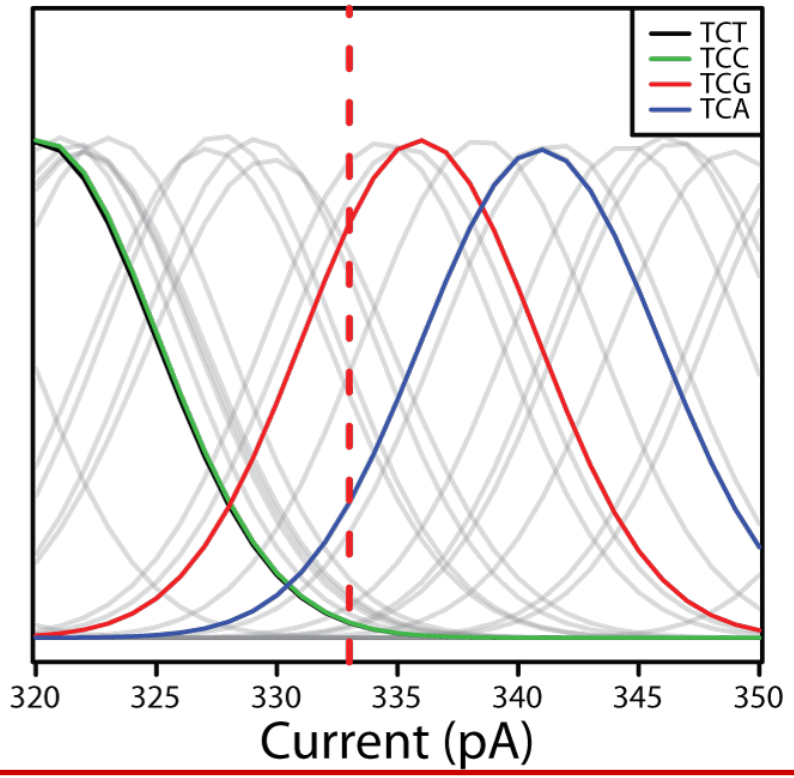
- Markov chains represent a series of states occurring in sequence, with defined transition probabilities. In a hidden Markov model the state is only observed indirectly.
- As an example, consider inferring the weather (without going outside) by observing whether people coming in from outside are wearing gloves or not

Prior Information for Decoding







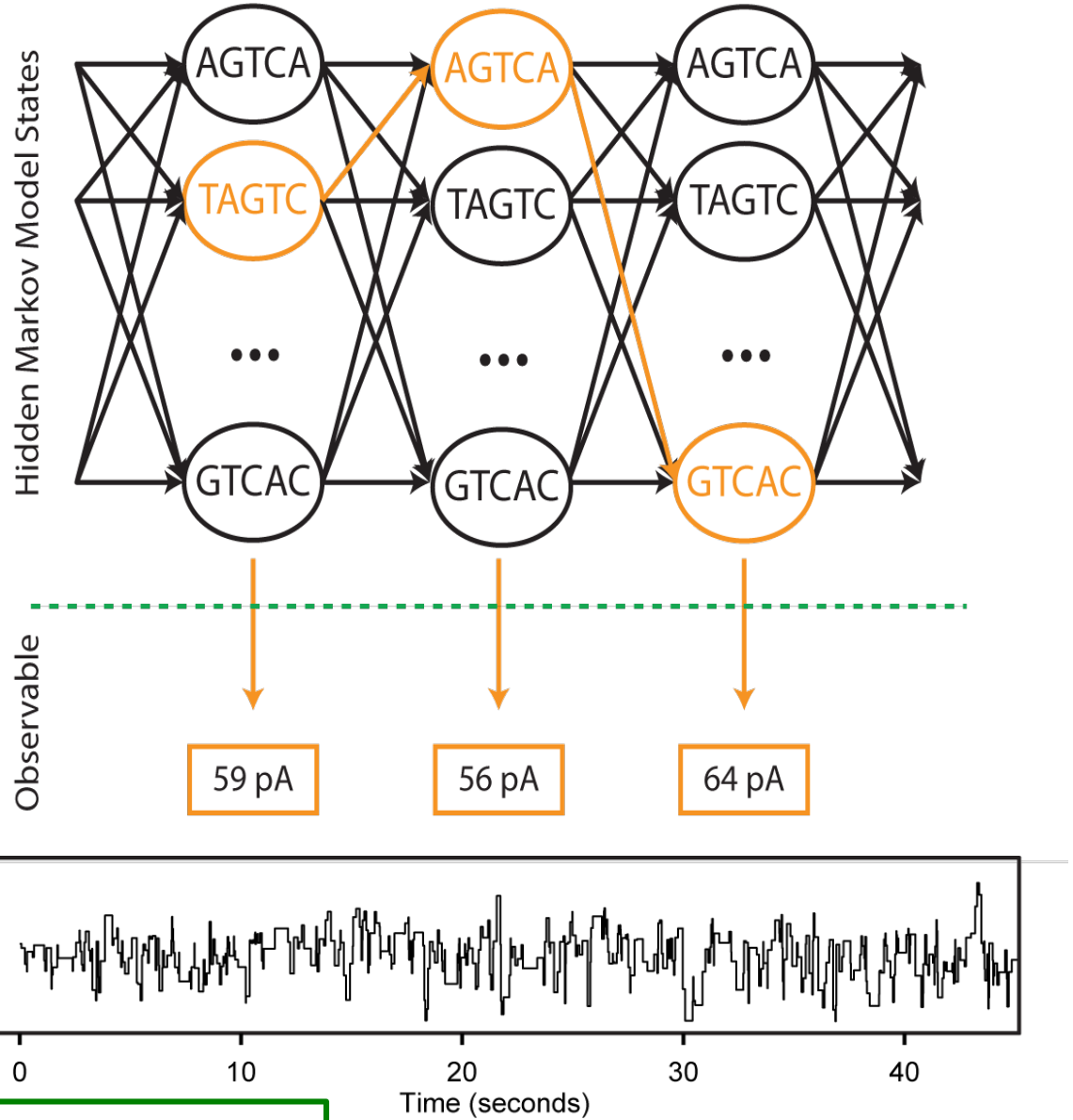


- With no prior information, a given current value may not be called correctly (333pA would be called as GGG)
- If we know the previous triplet, the next triplet is well defined, leaving only four possibilities, resulting in the correct call of TCG

Timp, et al. *Biophys J.* 30, 349-353 (2012)

Nanopore HMM basecalling

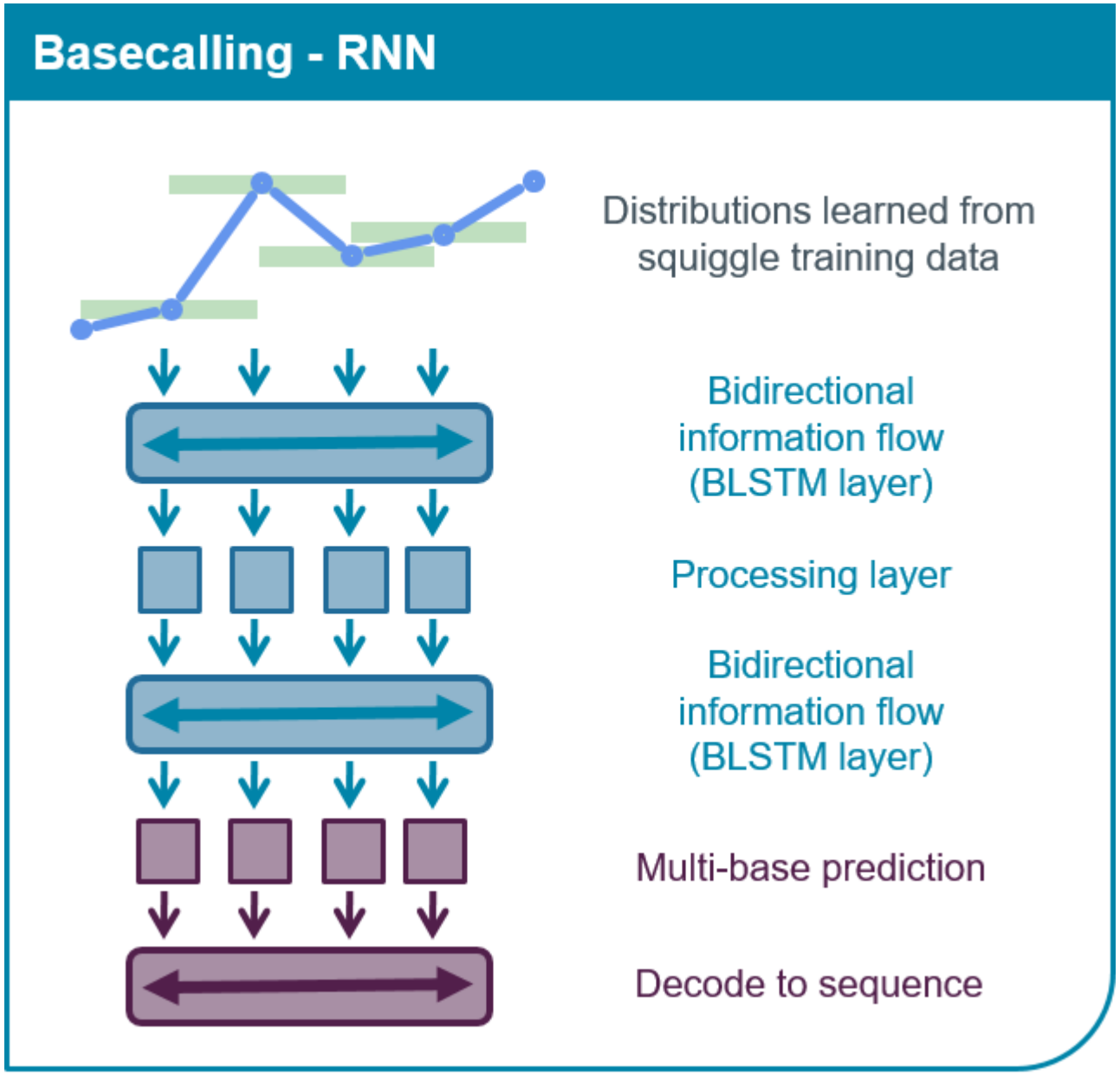
- By using a sequence of observables and maximizing the total joint probability given below, we find the sequence of states.
- This is done using the Viterbi algorithm – which grows, finding the most likely path for each step, saving the probabilities, to avoid recalculation.
- 1st generation basecallers from Oxford used a HMM for basecalling similar to the one detailed in our Biophysical paper
- Transition probability matrix for oxford seems to allow for a 0, 1 (most common), 2, or 5 (reset) move.
- We think that Oxford trained its basecalling model on unmethylated lambda



$$\delta_k \prod_t P(I(t) \mid k_t) \times T_{(t-1)(t)}$$

Basecalling shifting to RNN

- Recently (over the past year) there has been a shift to neural network based basecalling
- A *recurrent* neural network is still one with memory, that has a dependence on past computations
- Specifically two layers of Bidirectional Long Short Term Memory (BLSTM)
- These still require the same “training” data to learn what current distributions correspond to which k-mers – and the results are still k-mer based, as multiple bases still influence the current.



Building Genomes: Genomic Technology Development

- We wanted to try this out to see how well we could build genomes now
- As part of a team with Steven Salzberg (JHU) and David Neale (UCDavis) we are trying to sequence the giant redwoods
- This is a hard problem! The genomes are big (3X or 10X human) and have lots of stretches that are hard to distinguish

Sequoia sempervirens
Coast redwood
California – central/north coast

Sequoiadendron giganteum
Giant sequoia
California – Sierra Nevada

Save The Redwoods LEAGUE®

Two species in our redwood genome project

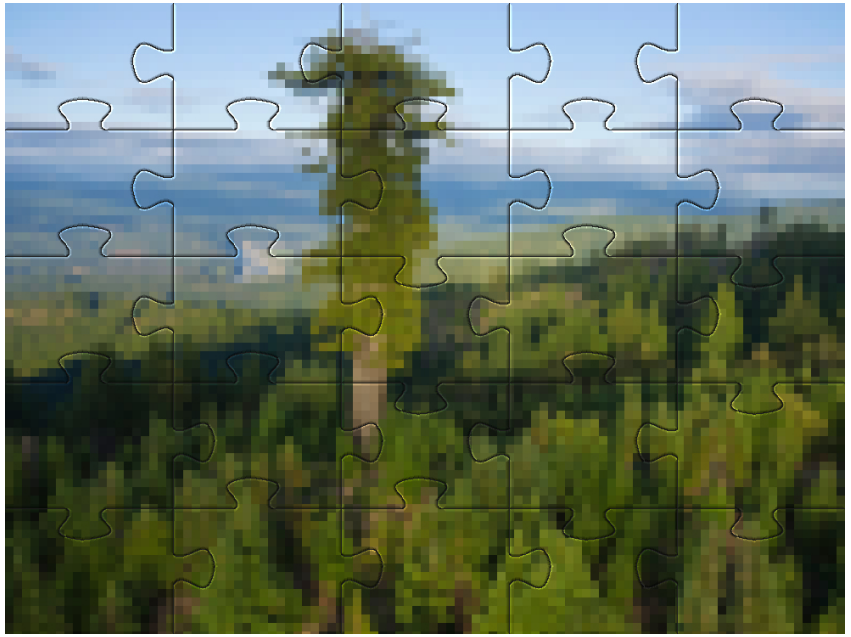
California endemics

Economic, cultural, and conservation value

”Advanced management strategies”

Huge genomes: 30Gb Coast, 9Gb Giant

Building Genomes: Genomic Technology Development


Building a genome is like putting together a puzzle:

- Larger (blurry) pieces = easier puzzle
- Small pieces = hard to put together, can't figure out "blue sky"
- Small + large pieces = cleary and easier to put together

Climbing & collection
by
Steve Sillett + team

DNA extraction
Neale lab

DNA extraction
Timp lab

Short read
sequencing @ UCD

Long read
sequencing @ JHU

Assembly
@ JHU

Reference
genome

Climbing & collection
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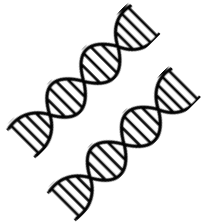
DNA
extraction
Neale lab

DNA extraction
Timp lab

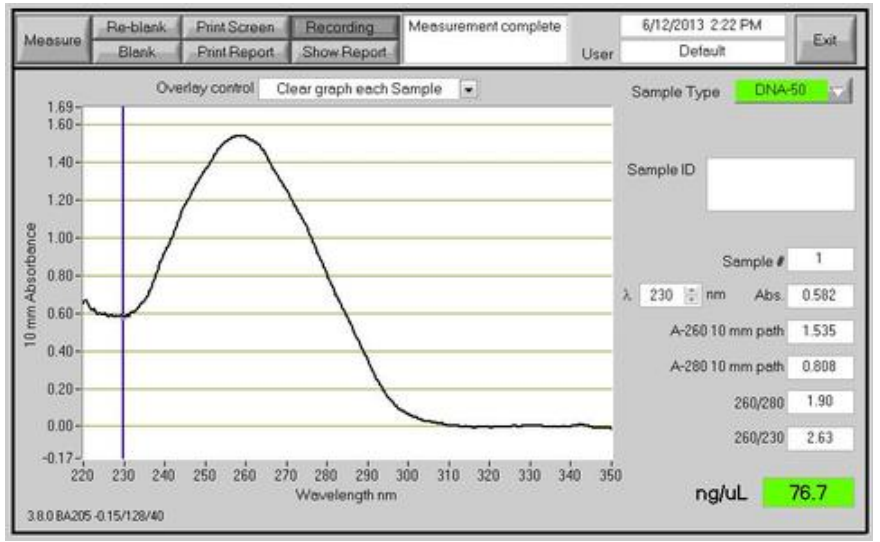


Short read
sequencing @ UCD

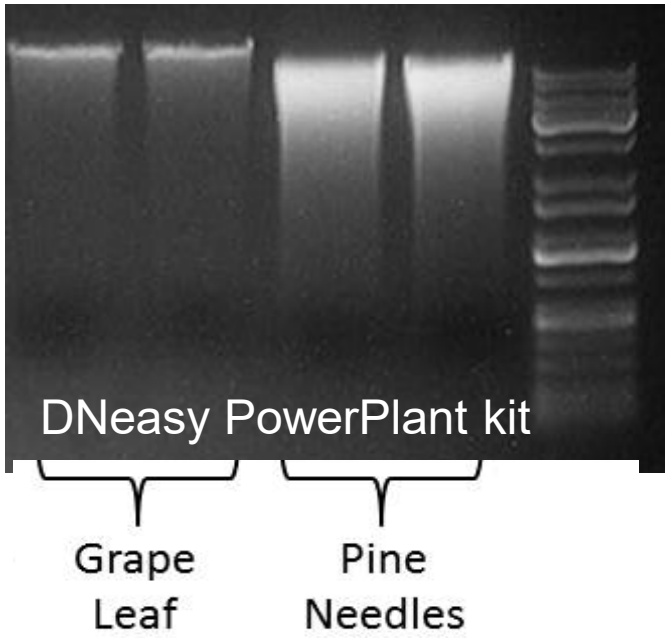
Long read
sequencing @ JHU

Problem

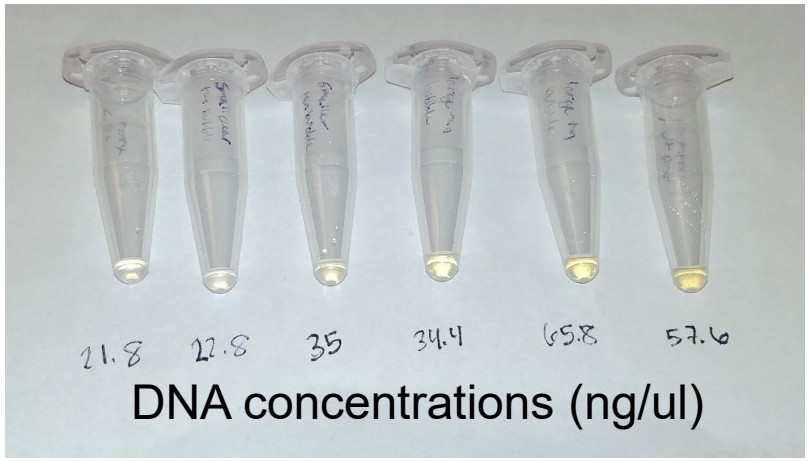
- Want: High molecular weight (HMW) DNA
  - 100kb + average
- Need: High yield
  - At least 10ug purified gDNA from 1 g of leaf tissue
- Need: High quality
  - Based on nanodrop spectra and gel migration
    - 260/280 ~1.8
    - 260/230 ~2.0+
    - No aberrant migration
- Reproducible/Robust protocol
  - Want no wizards
- High and consistent sequencing yield (- at least 3-5Gb per run)

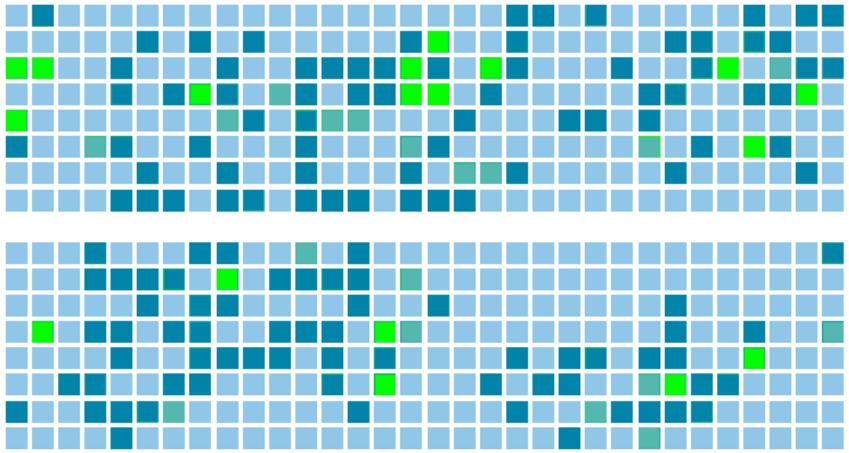


Sample realities before optimization



10kb

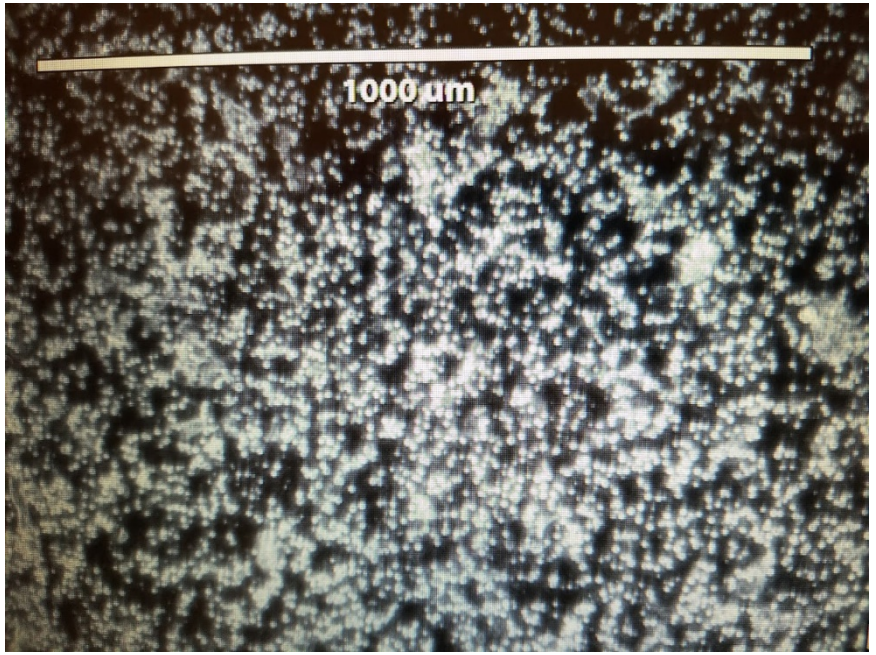


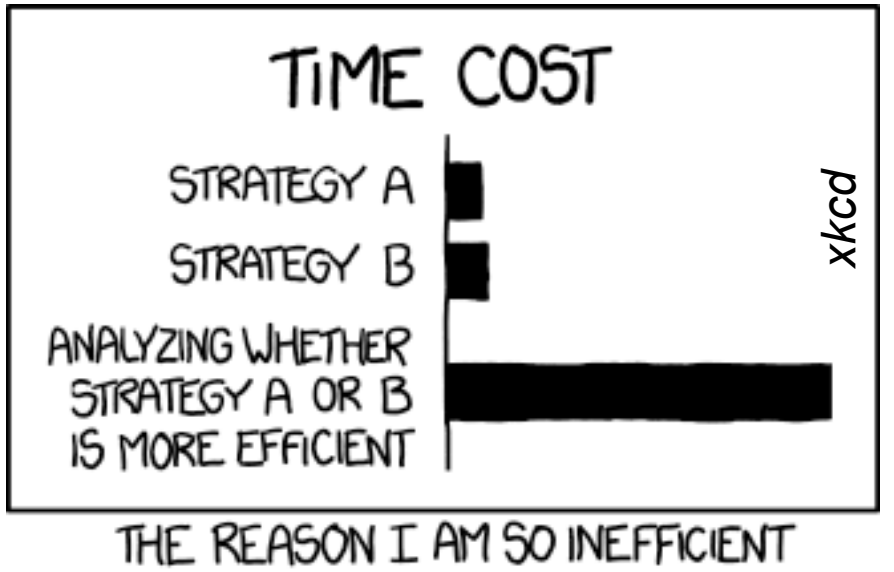


LMW	Low Yield	Poor Quality	Inconsistent	Low Seq Yield
<10kb average	<1ug gDNA Per 1g tissue	Residual polyphenolics and polysaccharides	Results varied largely by sample	<1Gb per run

Trials: DNA extraction

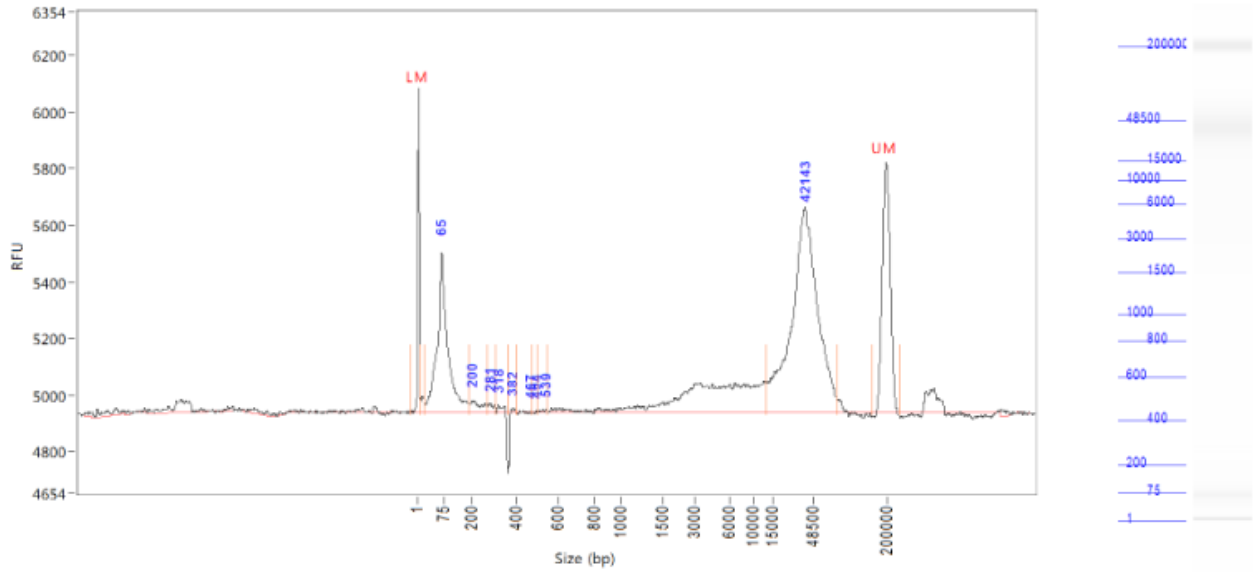
- Detergent (SDS, SLS, CTAB, Triton)
- Extract nuclei first (yes or no)
- Phenol chloroform (yes or no)
- Salt used for alcohol precipitation (NaCl, sodium acetate, ammonium acetate, none)
- Modifications for improving fragment lengths (agarose embedding, nanobind)
- Synchronous coefficient of drag alteration (SCODA) for purification



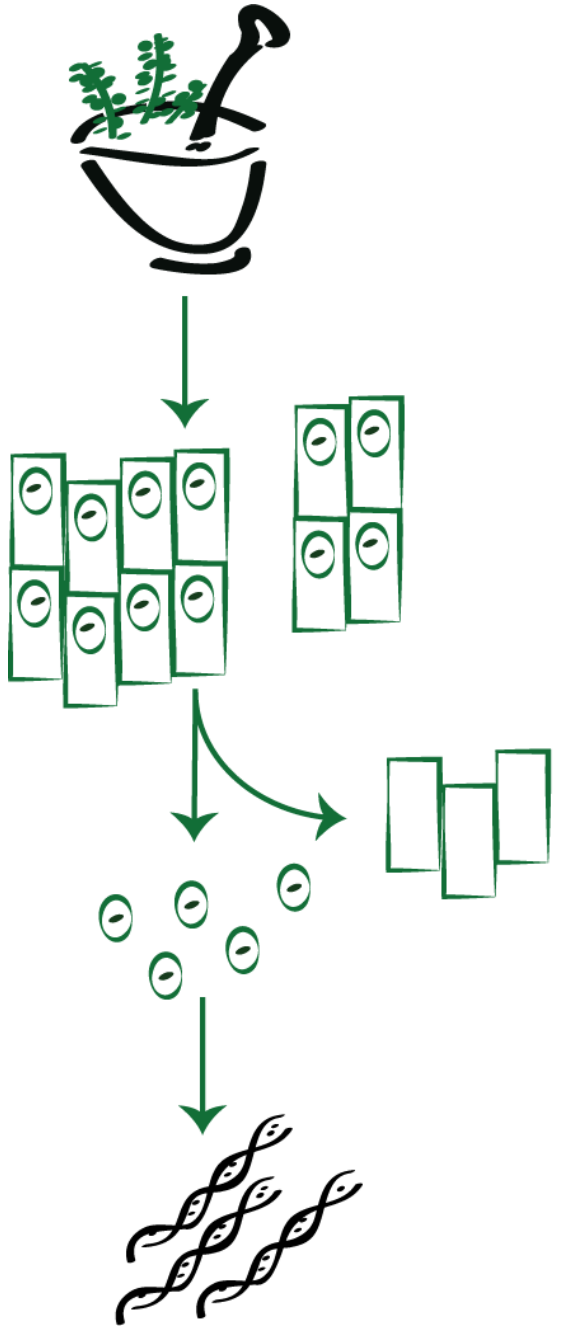


Top 3 extraction protocols: Zhang

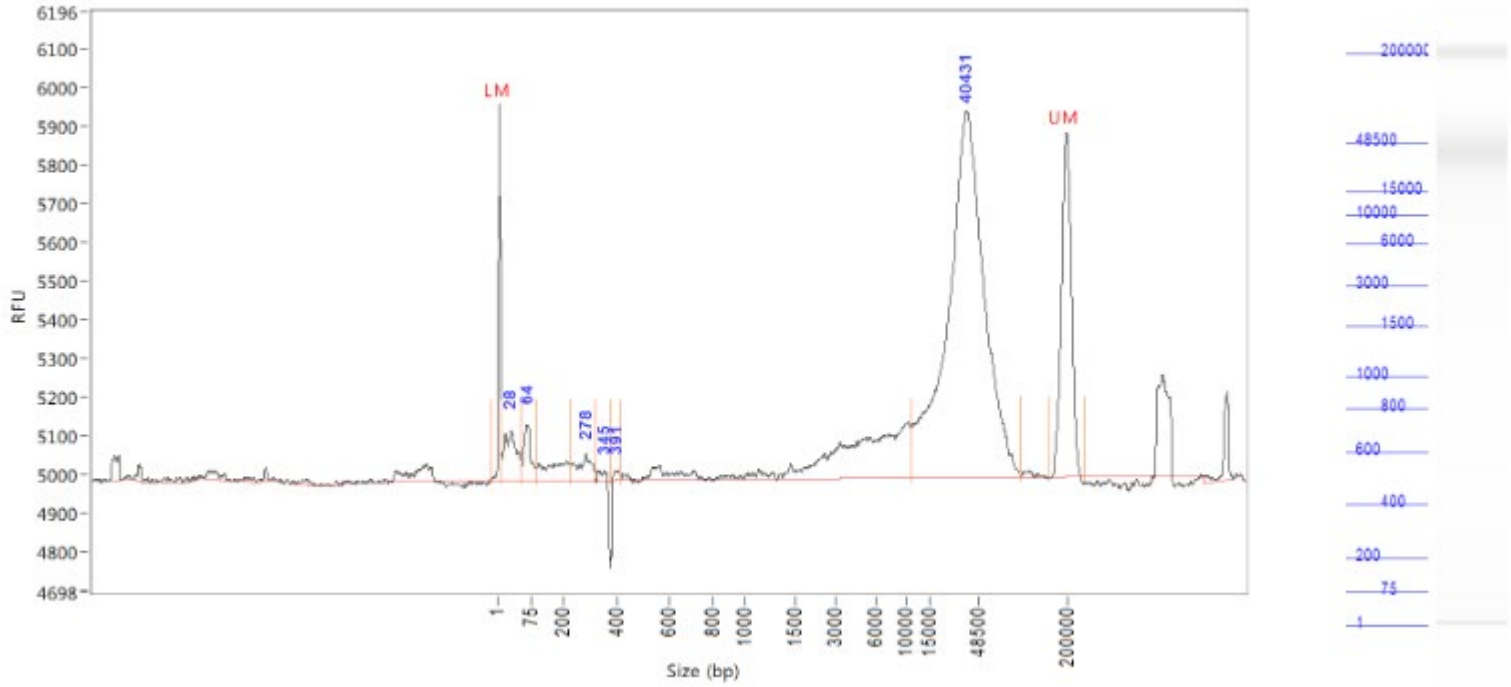
Sample: seq_zhang
Well Location: E5
Created: Tuesday, August 22, 2017 12:17:00 PM

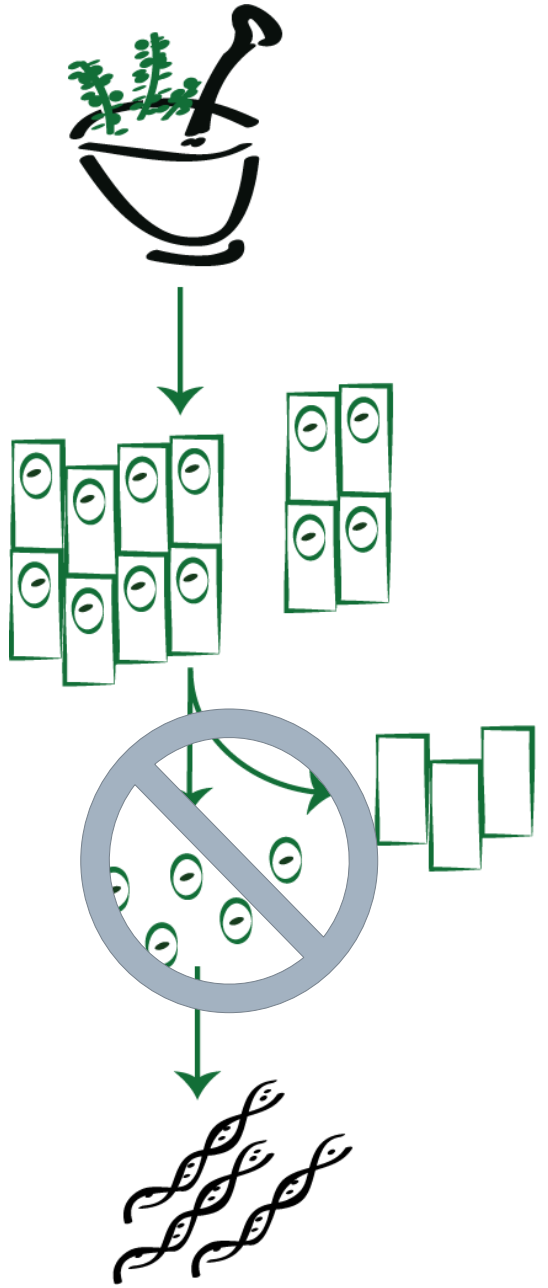


- Tissue ground in LN2
- Nuclei: Cell wall lysis -> filtration -> differential centrifugation
- DNA: Overnight SLS lysis, phenol chloroform, alcohol precipitation + sodium acetate



Top 3 extraction protocols: Healey

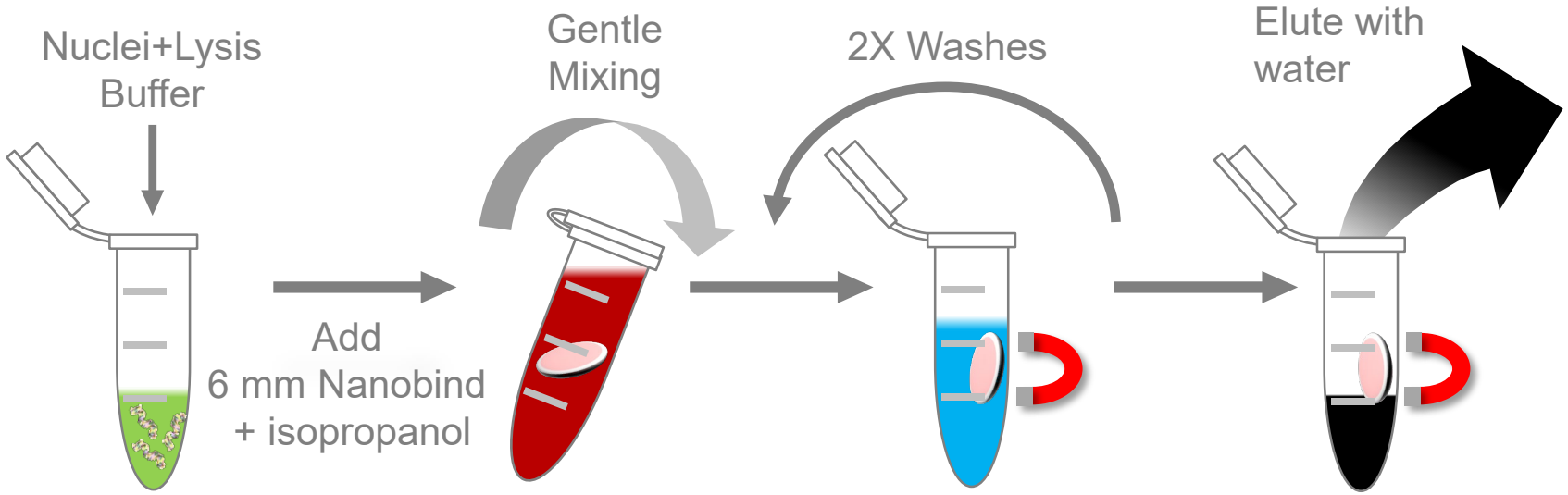


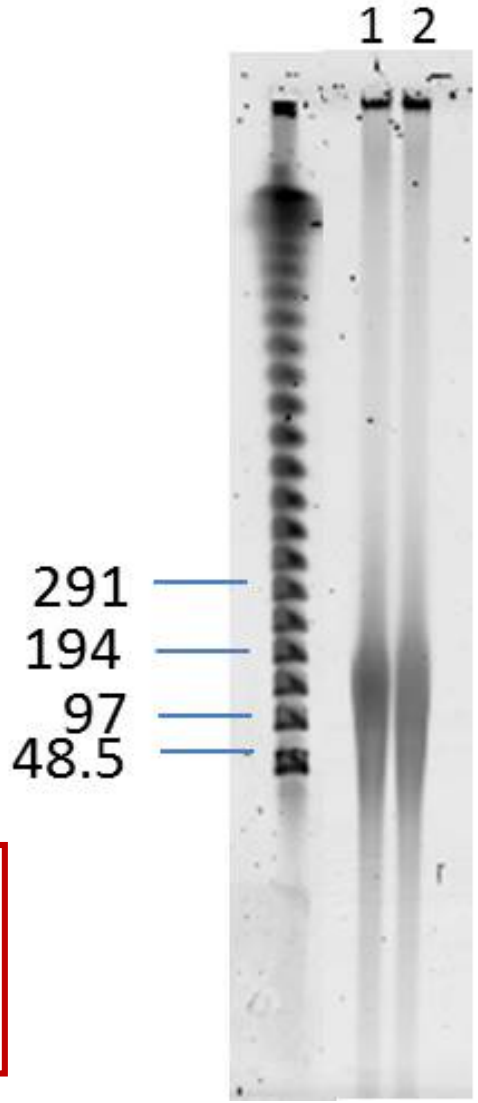


- Preferred plant protocol: Skips the differential nuclei extraction
- CTAB (cetyl trimethylammonium bromide) – cationic detergent
- CTAB extraction, chloroform, alcohol precipitation + NaCl, elute

Top 3 extraction protocols: Nanobind

Nuclei →

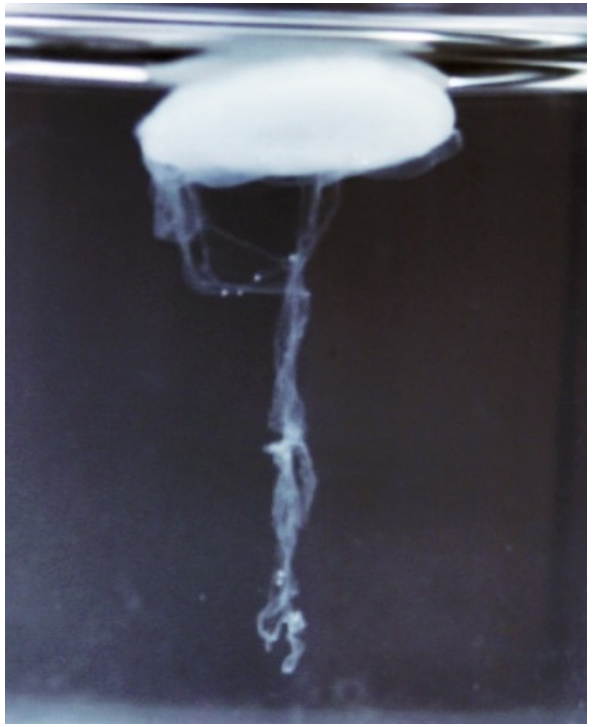




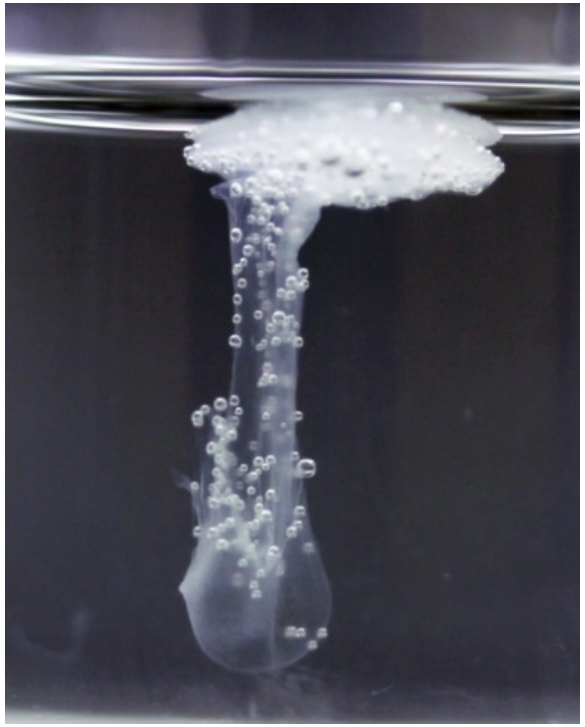
- Nanobind: Nuclei isolation as previously described
- Nanobind extraction with modified CTAB, EtOH washes, elute
- 45 minutes total time (after nuclei extract)

Unique Tentacle Binding Mechanism

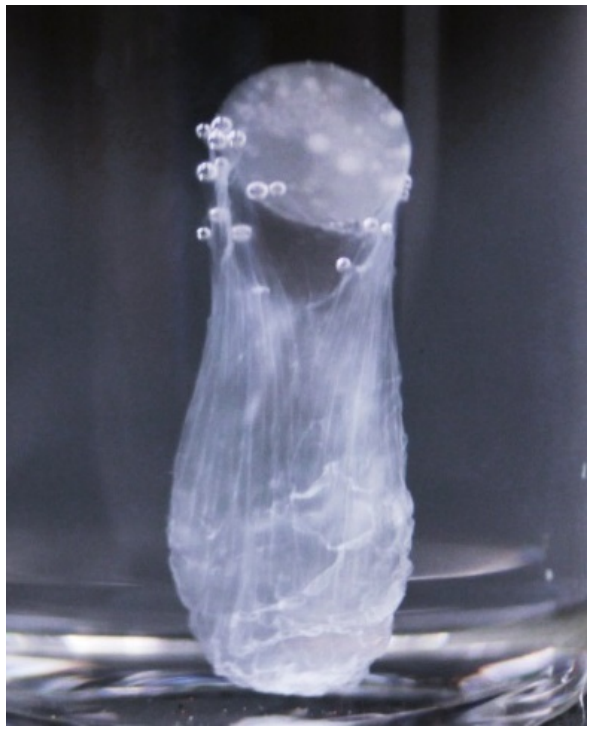
- Enhances binding capacity and protects DNA from shear forces



Low Input (10 µg)



Medium Input (50 µg)

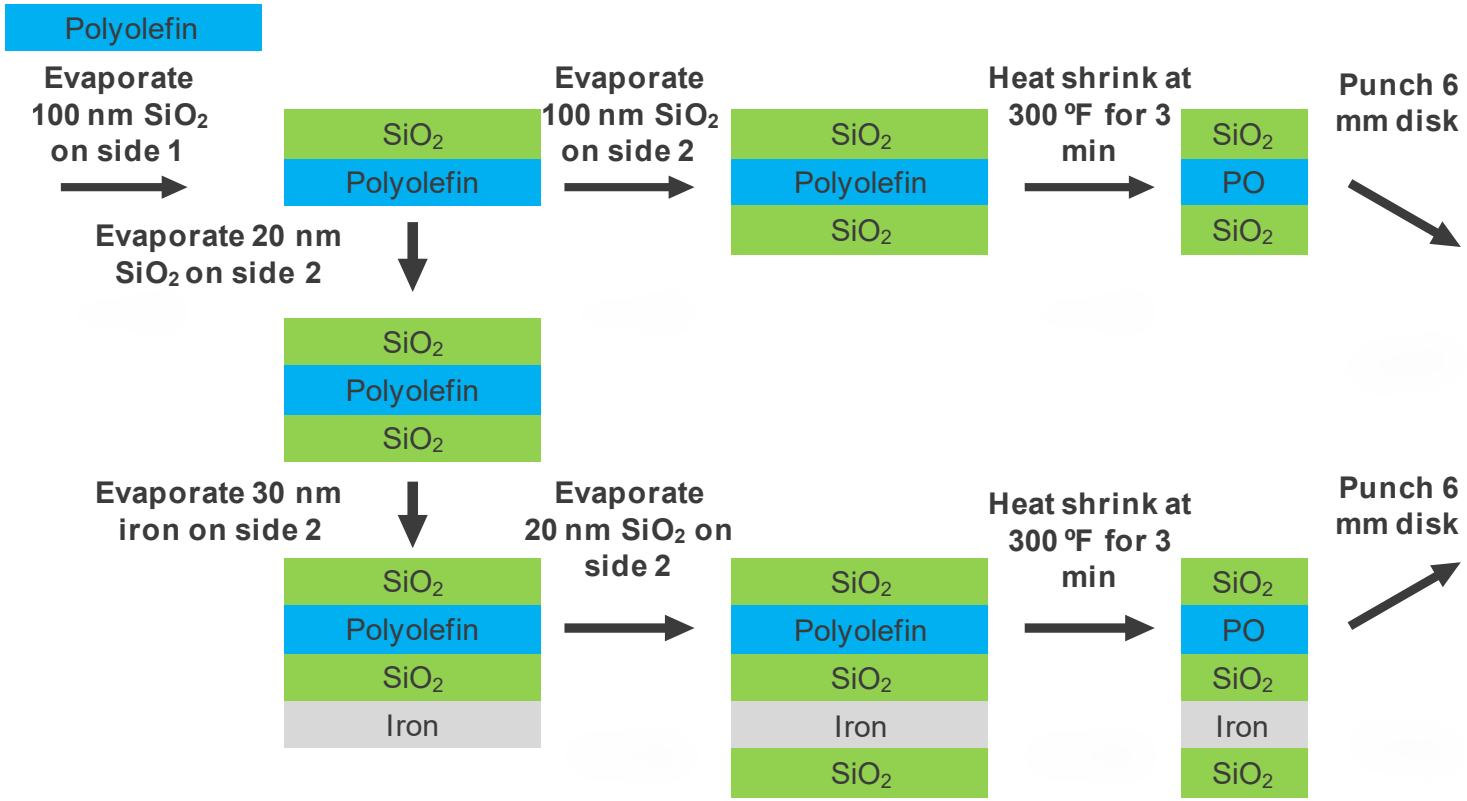


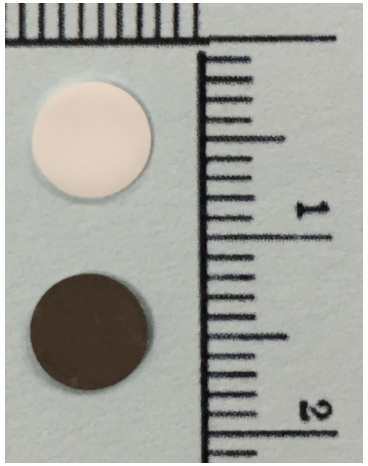
High Input (200 µg)

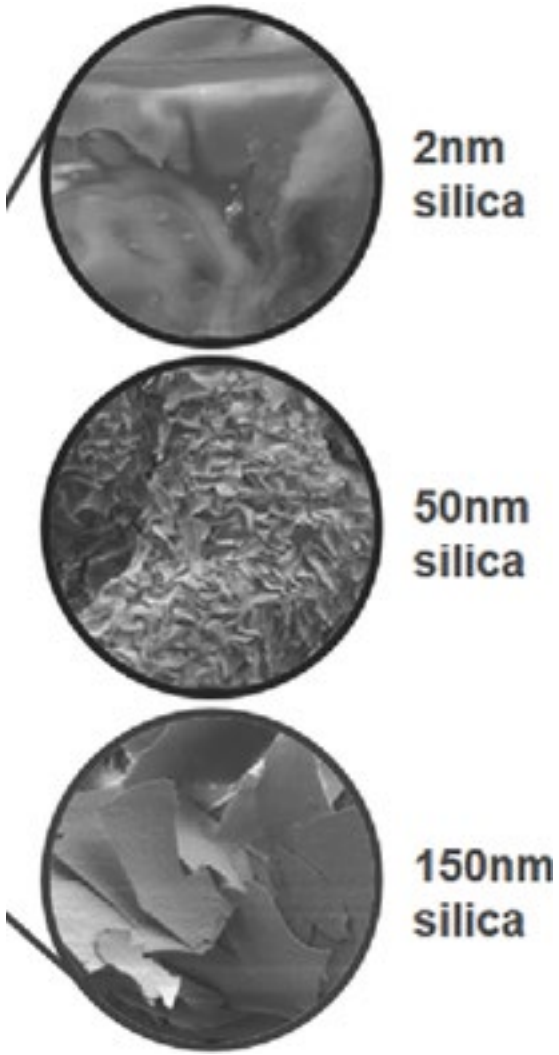
- Three material properties needed: low shear, non-porous, high surface area
- DNA tentacles form and extend from substrate to get high binding capacity
- Low shear unlike beads and columns

circulomics

Nanobind: How does it work





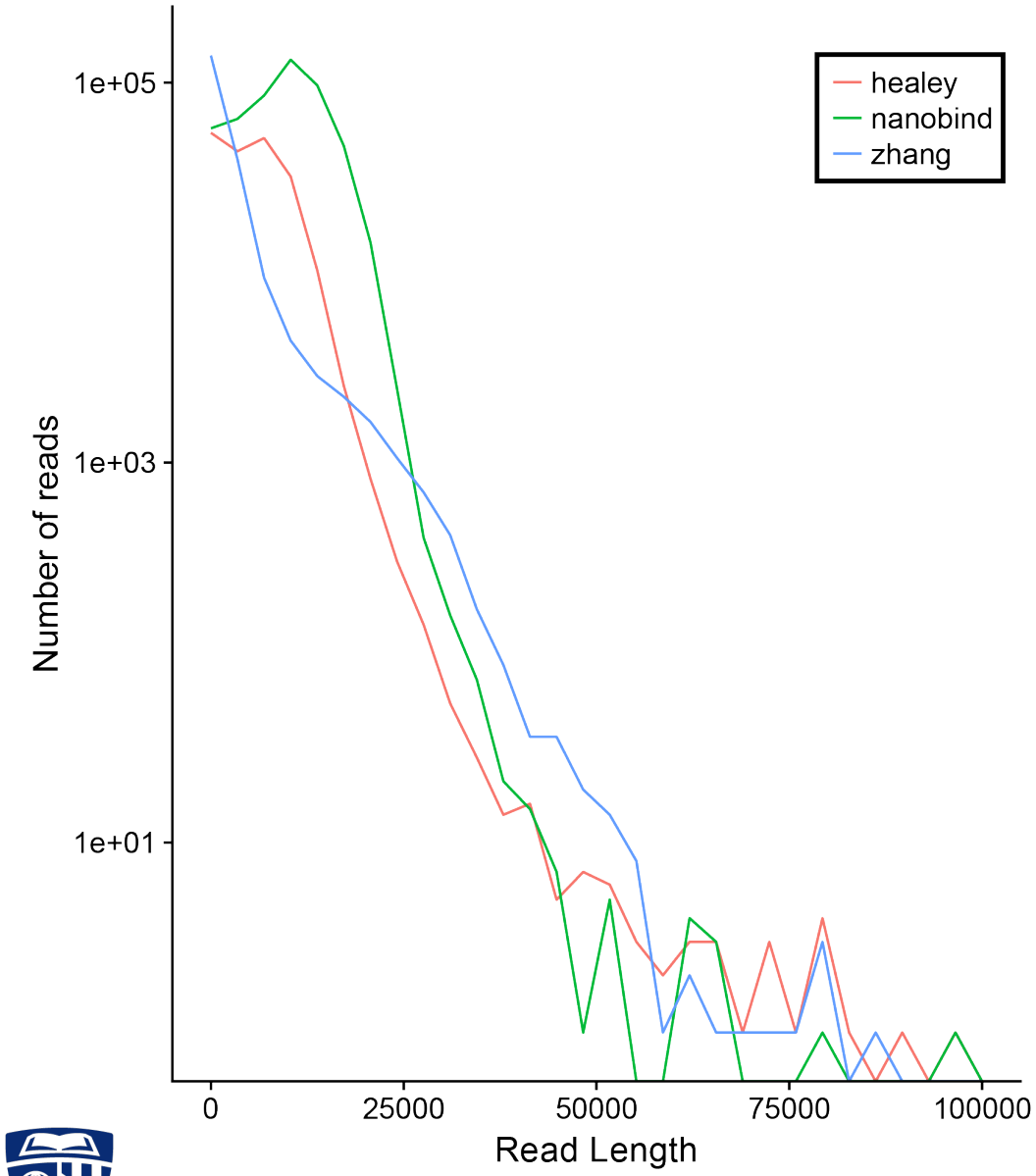


- Just nanostructured silica (SiO_2), essentially.
- With or without Iron layer for magnetism

circulomics

Zhang et al. Adv Mat. (2016)

Extraction Comparison



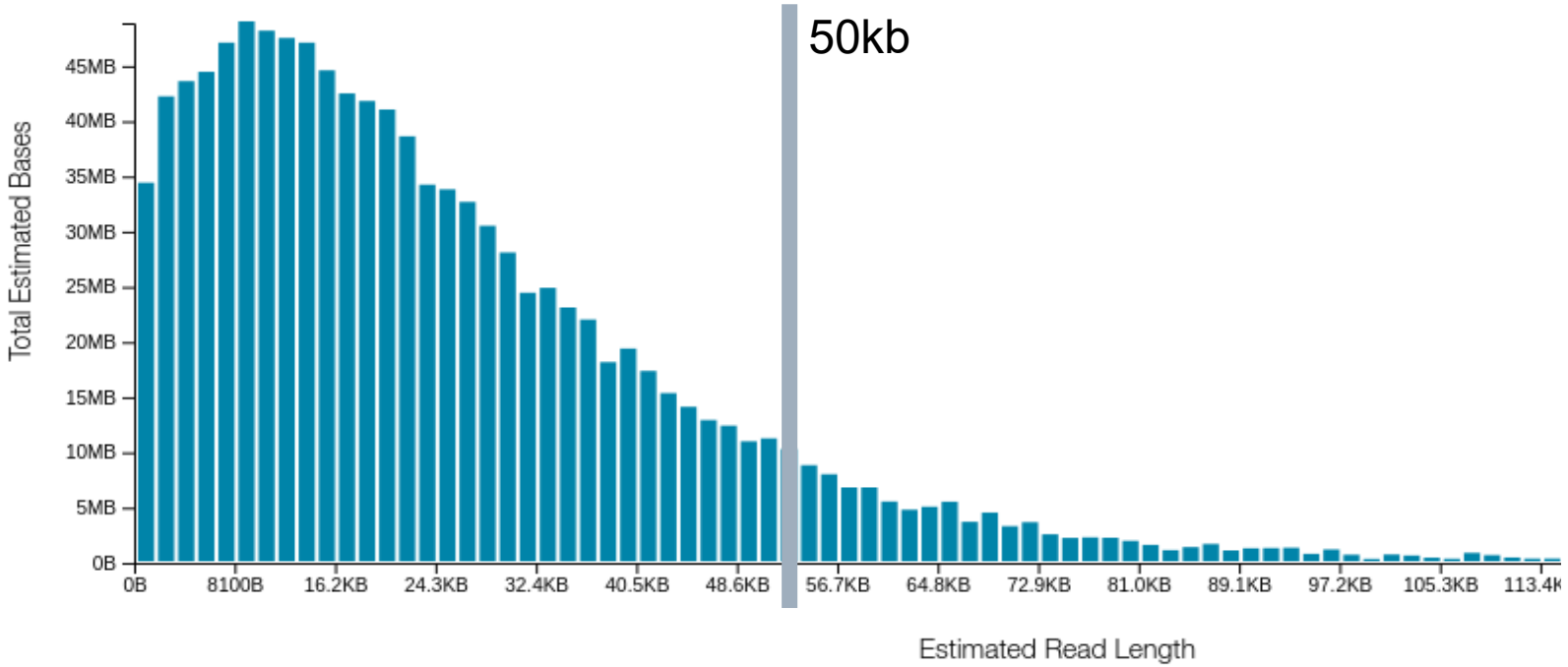
	Zhang	Nanobind	Healey
Reads	201k	500k	195k
Yield	0.51Gb	4.72Gb	1.08Gb
N50	7.1kb	12.3kb	8.6kb
Median	929	9.8kb	5.1kb

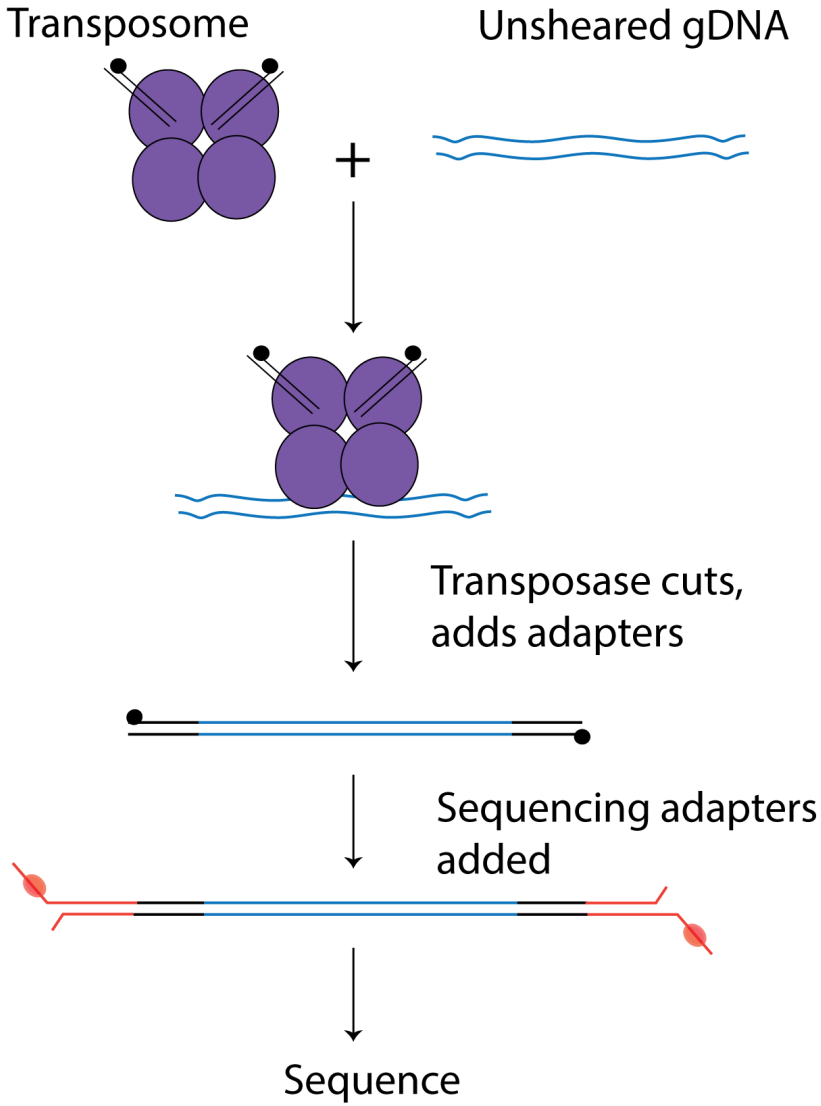
- Zhang seemed (in our hands) to fragment badly)
- Nanobind and Healey seemed to give reasonable read lengths, but doesn't match with PFGE profile size

Improving Read Lengths: Rapid kit RAD004

15 minute protocol

Yield:
3Gb from 150K reads
0.89Gb from >50kb reads



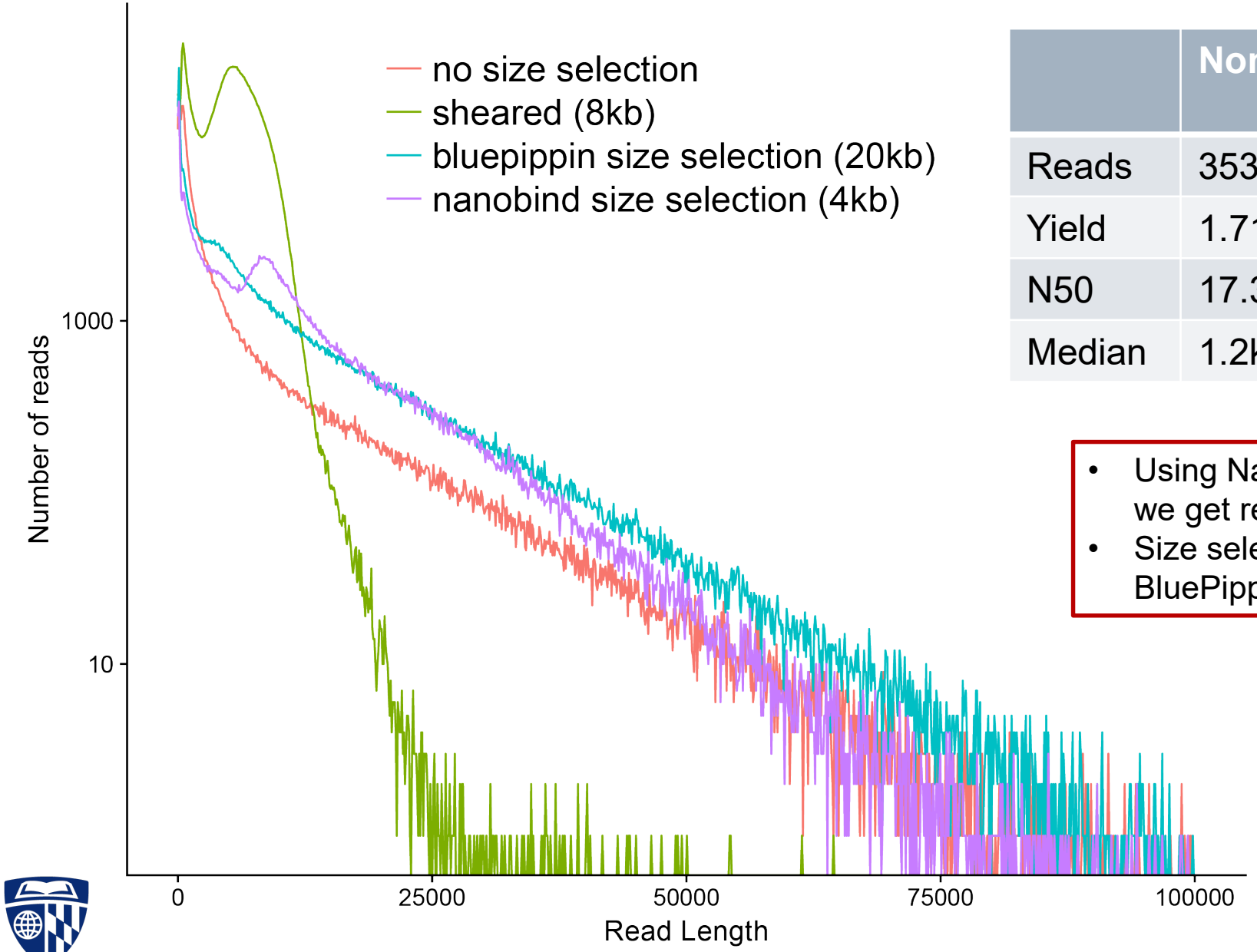


Improved extraction and sequencing methods affect assembly contiguity

Assembly with MaSuRCA 3.2.4+ scaffolding with HiRise(Dovetail genomics)					
	Sequence in contigs (Gbp)	**N50 contig (Kbp)**	**N50 scaffold (Kbp)**	**Number of contigs**	**Number of scaffolds**
Illumina only	7.9	12	65	2,507,175	1,007,217
Illumina+Nanopore	8.1	360	489	49,676	39,821
Illumina+Nanopore+ Chicago+Hi-C	8.1	318	689,571	52,835	8,215

- Improvements in contig and scaffold size over other conifer assemblies afforded by long reads
- MaSuRCA assembler (Zimin et al, Bioinf 2013; http://www.genome.umd.edu/masurca.html)

Size Selection – Going even longer

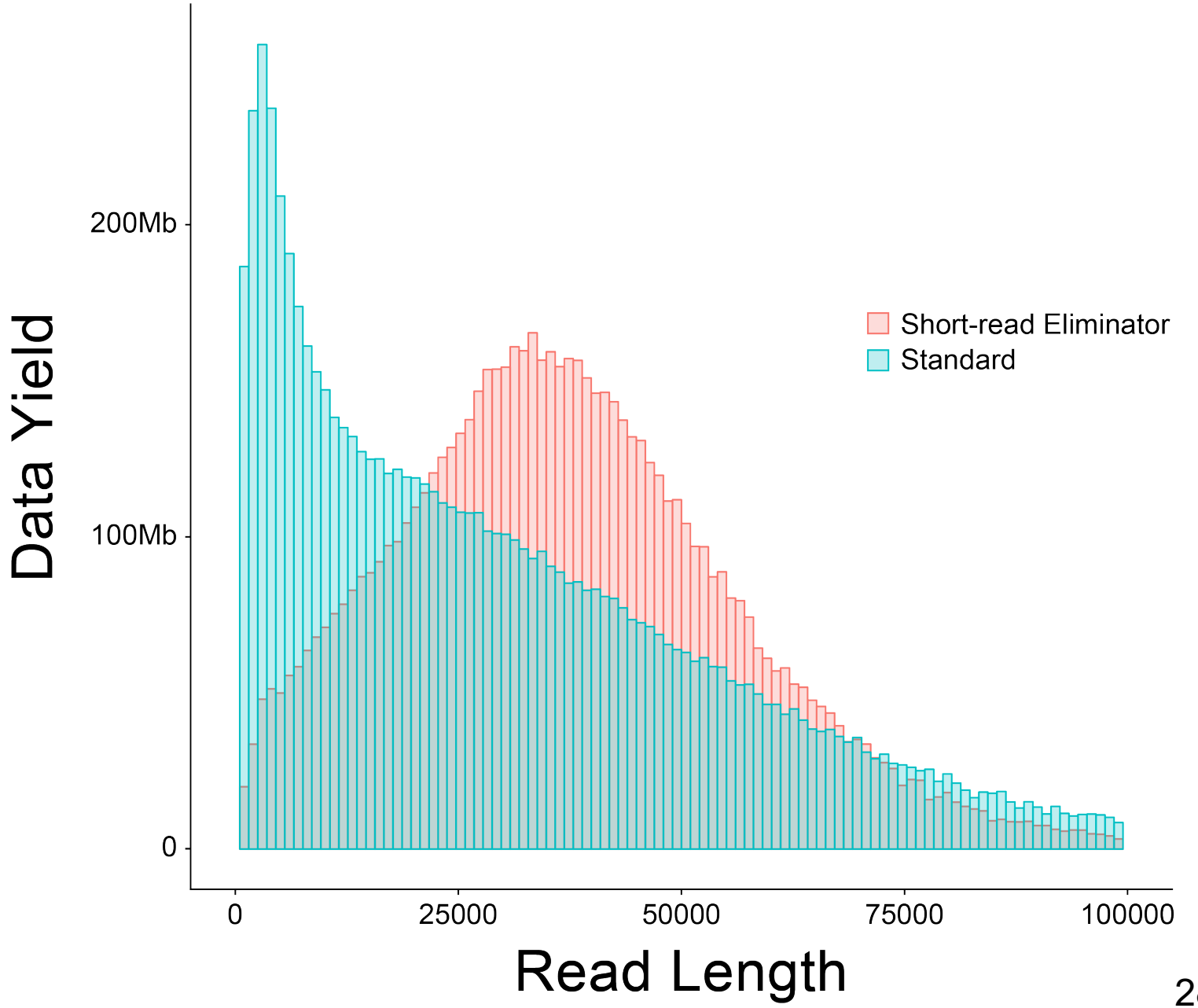


	None	Sheared	BP (20kb)	NB (4kb)
Reads	353k	2060k	435k	400k
Yield	1.71Gb	10.1Gb	3.65Gb	3.57Gb
N50	17.3kb	6.6kb	19.0kb	15.7kb
Median	1.2kb	5.1kb	4.3kb	6.8kb

- Using Nanobind+PEG as an AMPure replacement, we get reasonable yield
- Size selection for long reads a little worse than BluePippin, but more >5kb reads

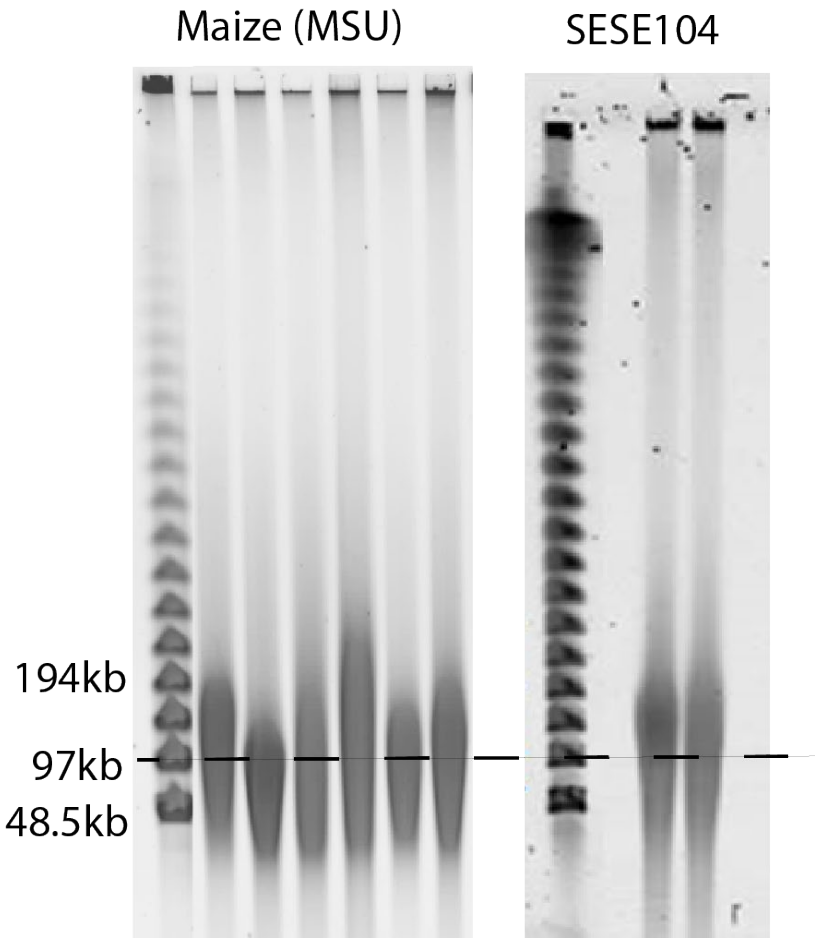
Read Length

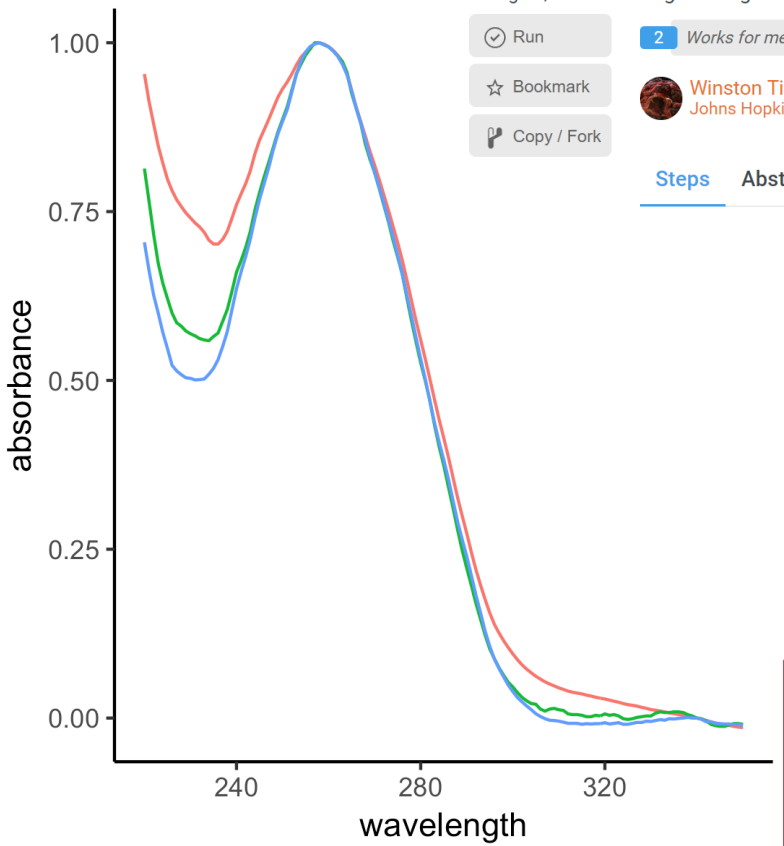
- Working with Circulomics we have been trying to get the read length up
- Using a size selection with their Nanobind material, read N50 can be substantially improved
- There is still room for improvement often still difficult to get both high yield and high read length

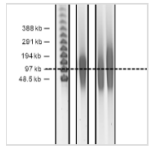


circulomics

Methodology extensible to other plants







Aug 07, 2019

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High Molecular Weight DNA Extraction from Recalcitrant Plant Species for Third Generation Sequencing

Rachael Workman[1], Renee Fedak [2], Duncan Kilburn[3], Stephanie Hao[3], Kelvin Liu[2], Winston Timp[3]

[1]Johns Hopkins University, [2]Circulomics, Inc., [3]Johns Hopkins University, Department of Biomedical Engineering

2 *Works for me* dx.doi.org/10.17504/protocols.io.4vbgw2n

Winston Timp
Johns Hopkins University

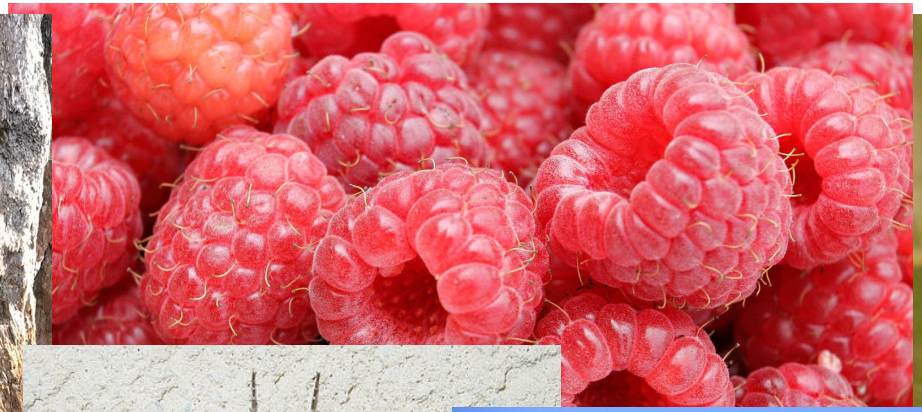
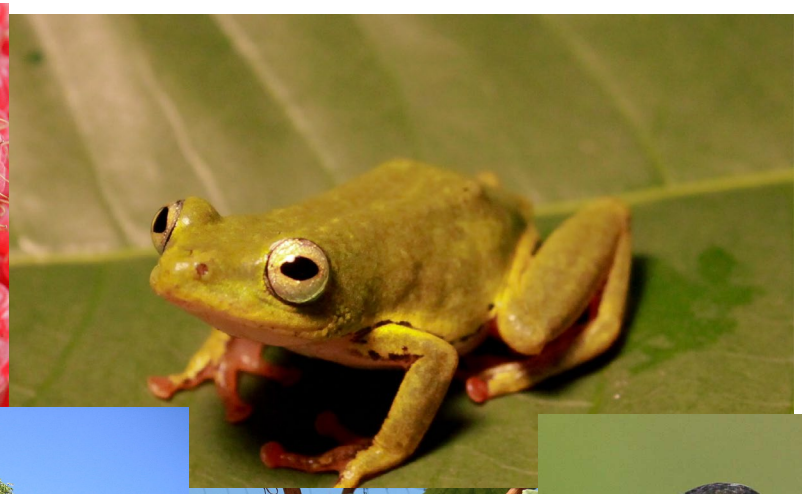
Steps Abstract Guidelines Warnings Materials Metadata Metrics

sample
SESE104
SEGI21
Maize

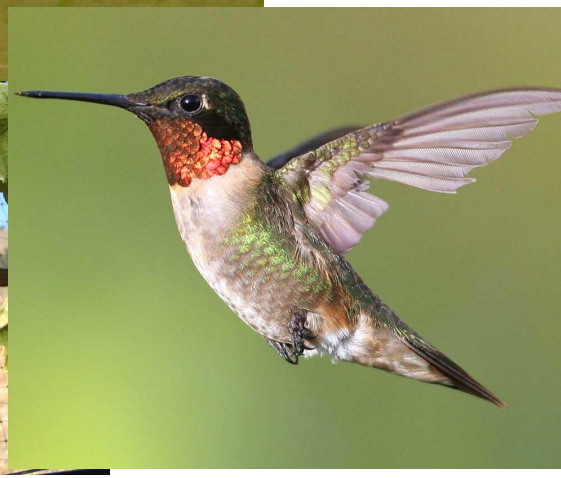
- Protocol is online at protocols.io
- Nanobind is available from Circulomics

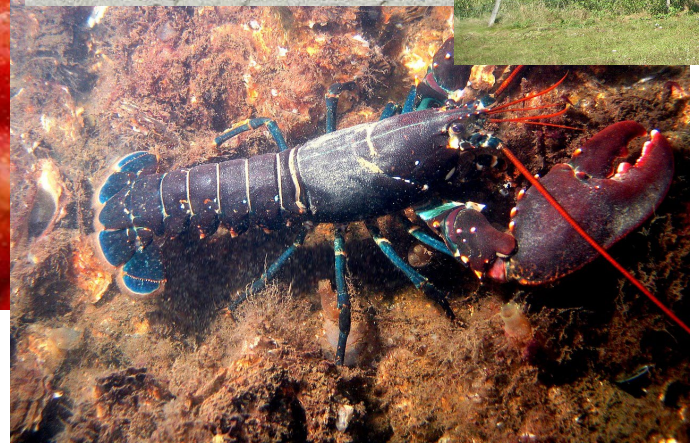
Maize results courtesy of B. Vaillancourt and Krystle Wiegert-Rininger of the C. Robin Buell Lab at Michigan State University

Building Genomes: Genomic Technology Development

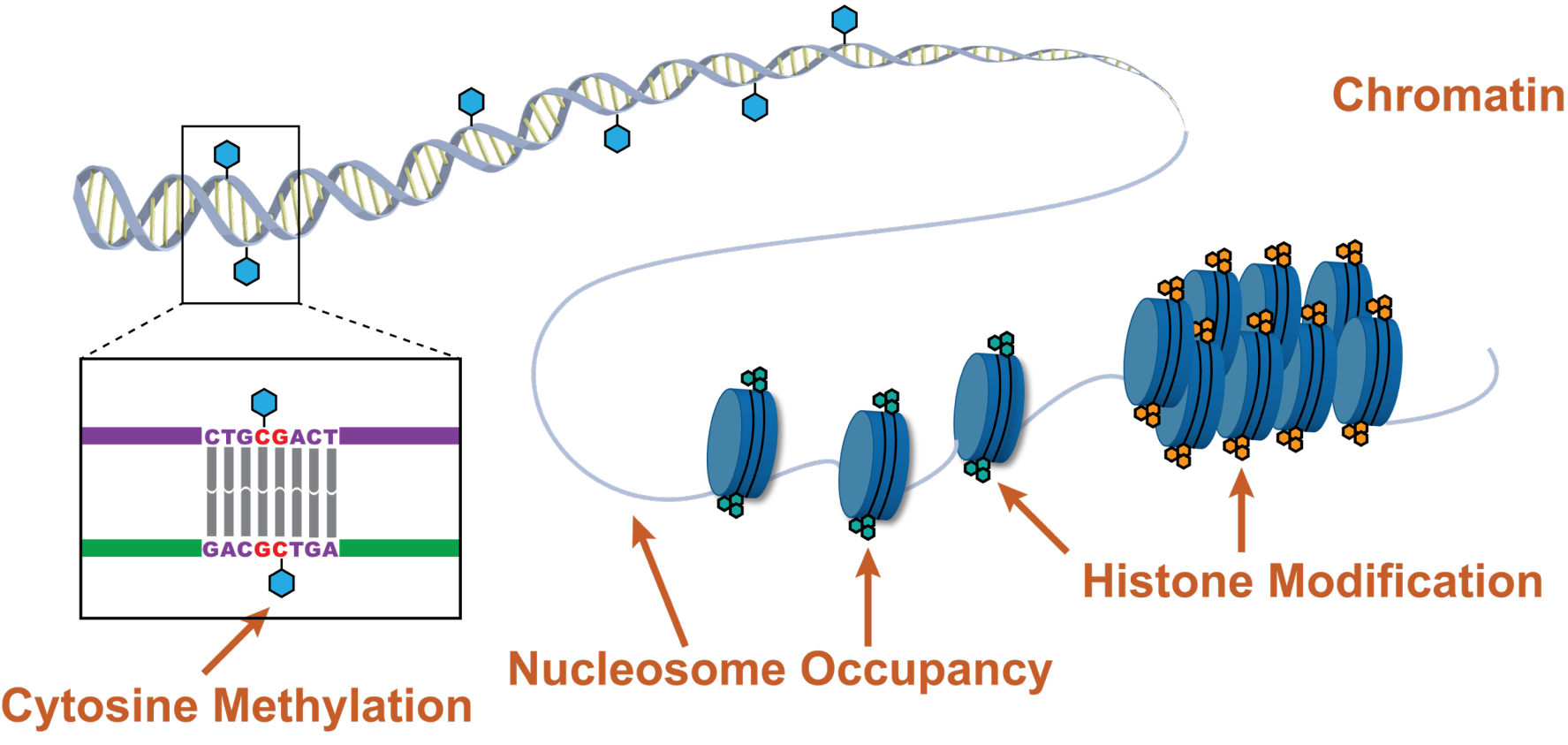


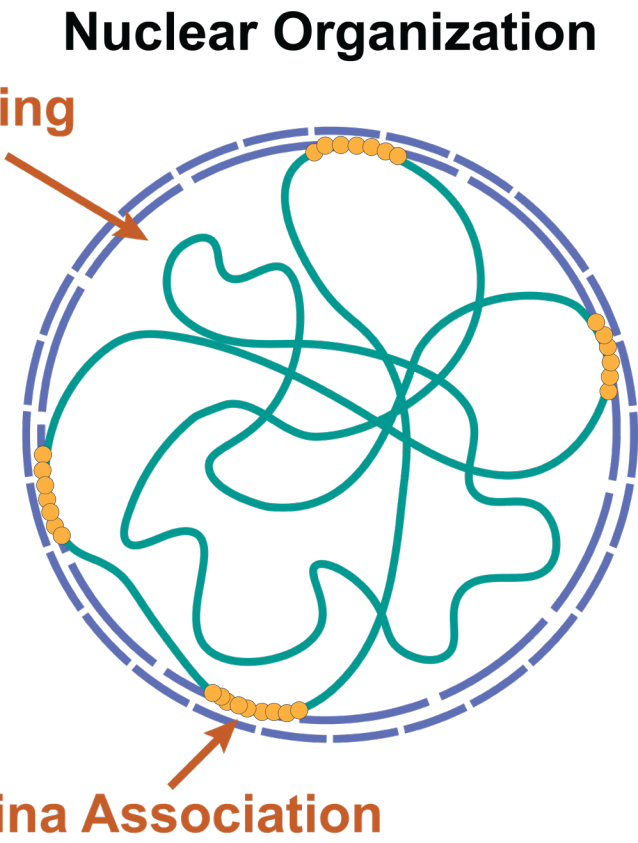




- Long reads are great to build genomes of non-model organisms!
- Working with collaborators here and across the world, we are finding new ways to extract and generate genomes from these organisms – from moths to hummingbirds to giant sequoia

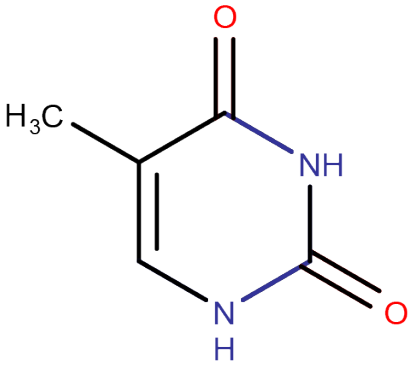
Epigenetics: Modern



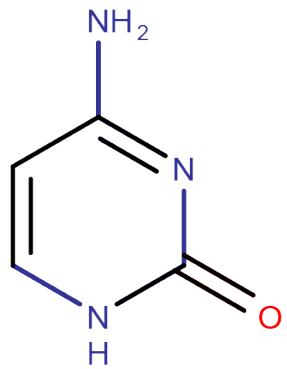


- Modern Definition of epigenetics involves heritable changes other than genetic sequence, e.g., positive feedback, high order structure, chromatin organization, histone modifications, DNA methylation.
- An analogy to a computer system:
  - DNA Sequence = Hardware
  - User input = Environment
  - Systems Biology = Running programs
  - **Epigenetics = RAM**

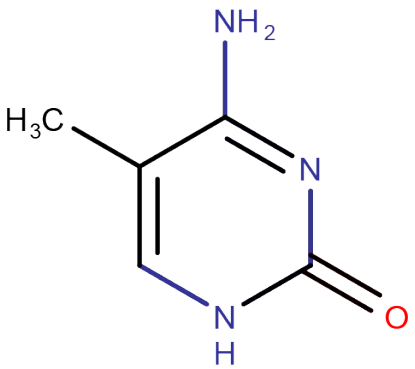
Nanopore Sequencing of Modifications



Thymine

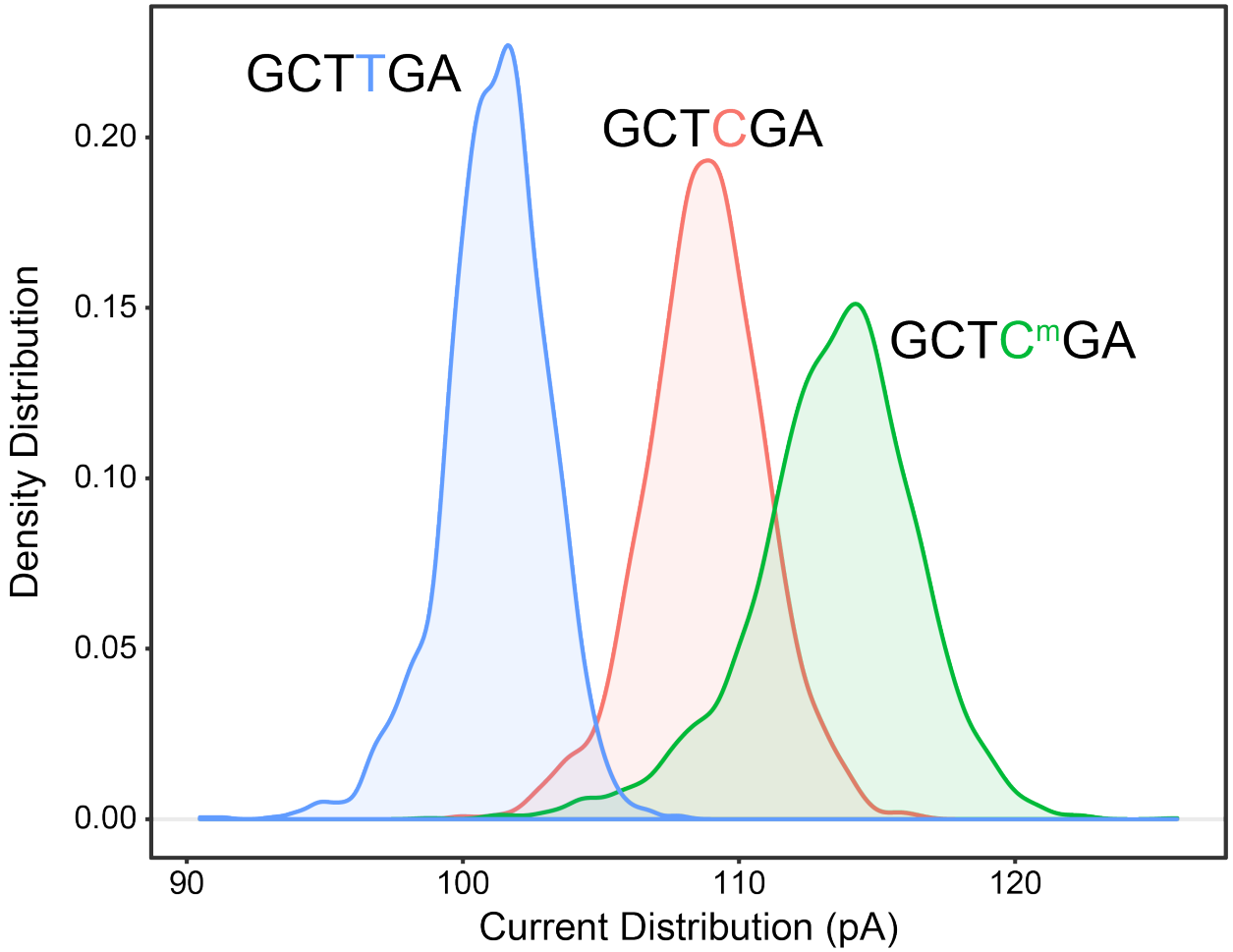


Cytosine



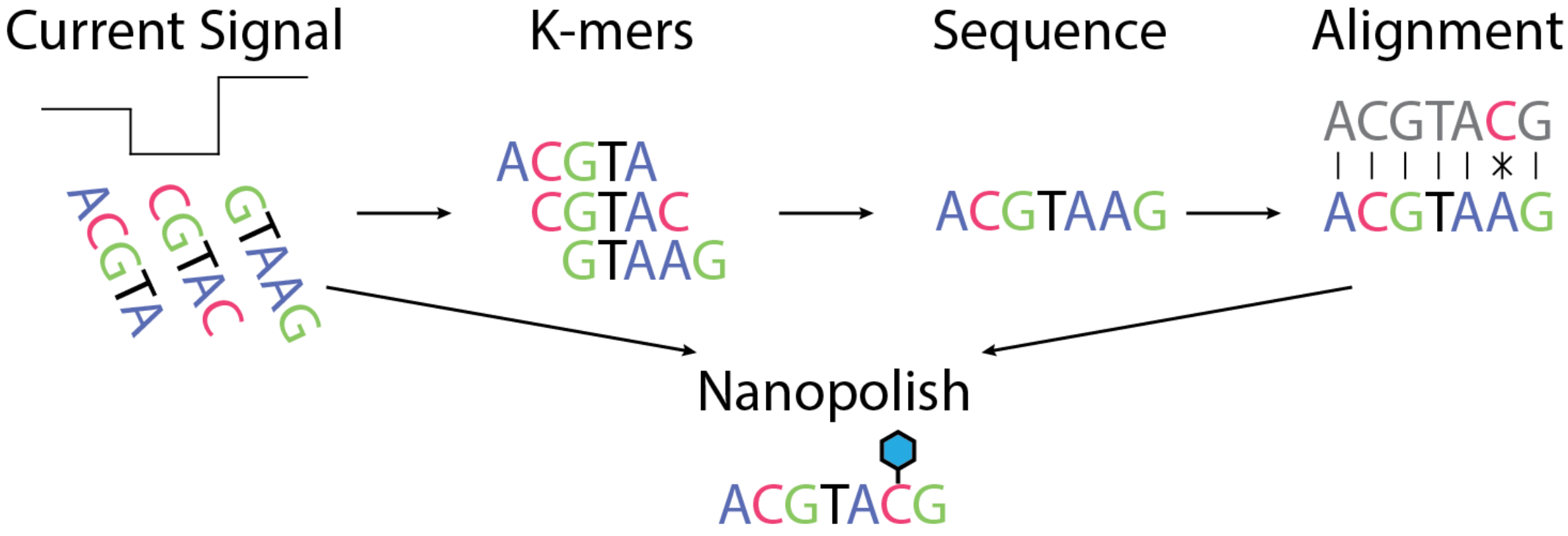
5-methylcytosine

- To generate methylated samples, we treat unmethylated DNA (PCR amplified E. Coli gDNA) with M. SssI methyltransferase
- Distributions of observed current for GCT[T/C/mC]GA demonstrate the type of signal between methylated and unmethylated k-mers



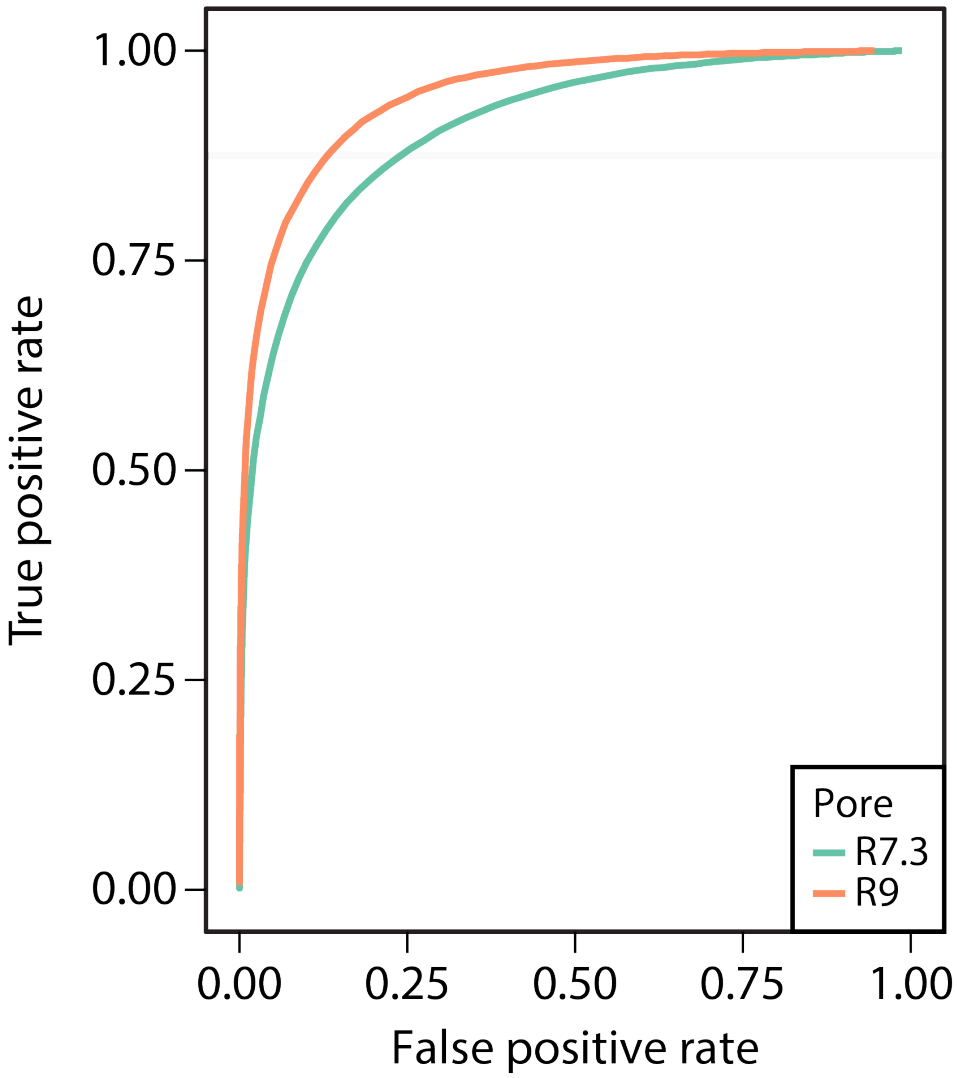
Simpson et. al *Nature* Methods 2017

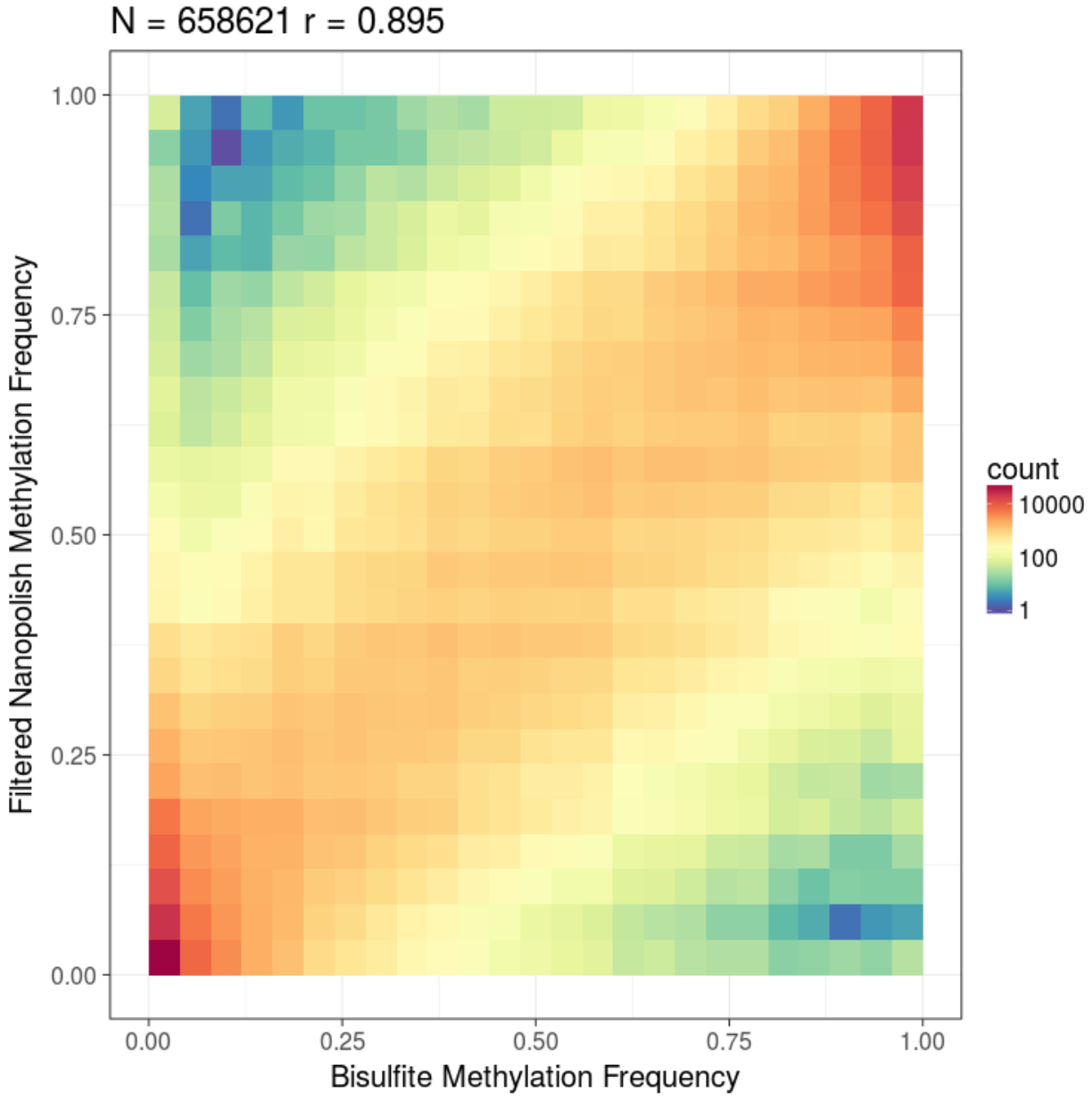
Nanopore: nanopolish methyltrain



- With *nanopolish* we can call the probability: $\frac{P(\mathcal{D}|S_m)}{P(\mathcal{D}|S_r)}$
- Where S_m is the probability methylated for a given observable D and S_r the probability unmethylated)
- We then take the log of this likelihood ratio, and threshold for >2.5 as methylated; <2.5 as unmethylated

Nanopolish Methylation

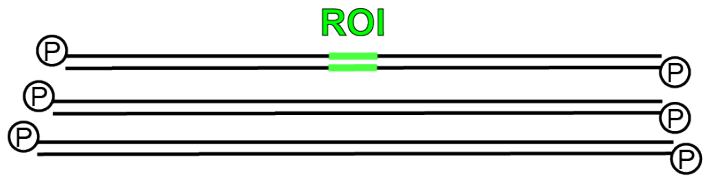




R9 calculates methylation 94% accurate at 77% of sites
NA12878 data shows .895 correlation with bisulfite

Jain et al *Nat Biotech* (2018)
Simpson et al *Nat Methods* (2017)

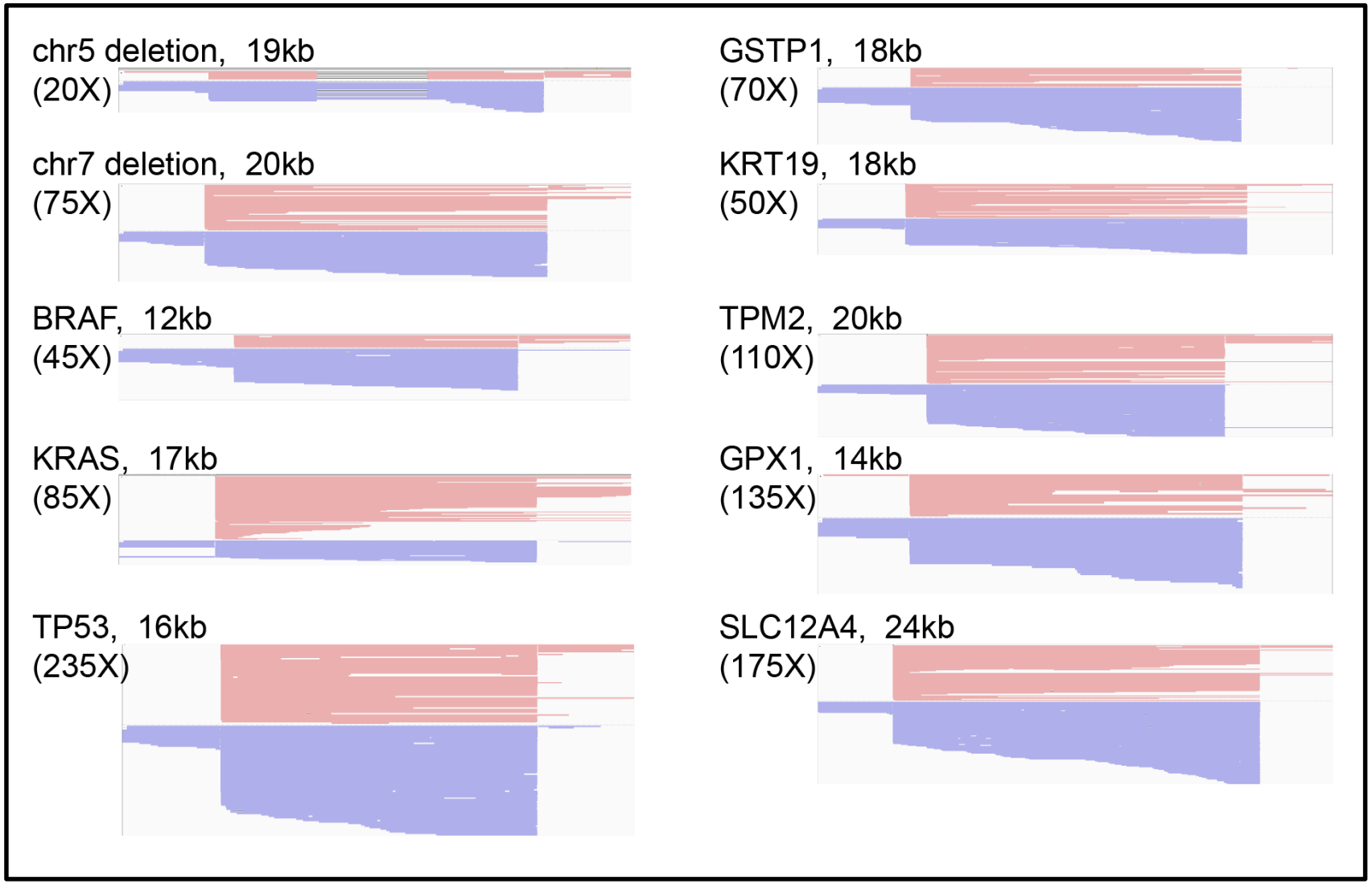
Cas9 enrichment Method



Gilpatrick et al bioRxiv (2019)

Using a panel of guideRNAs

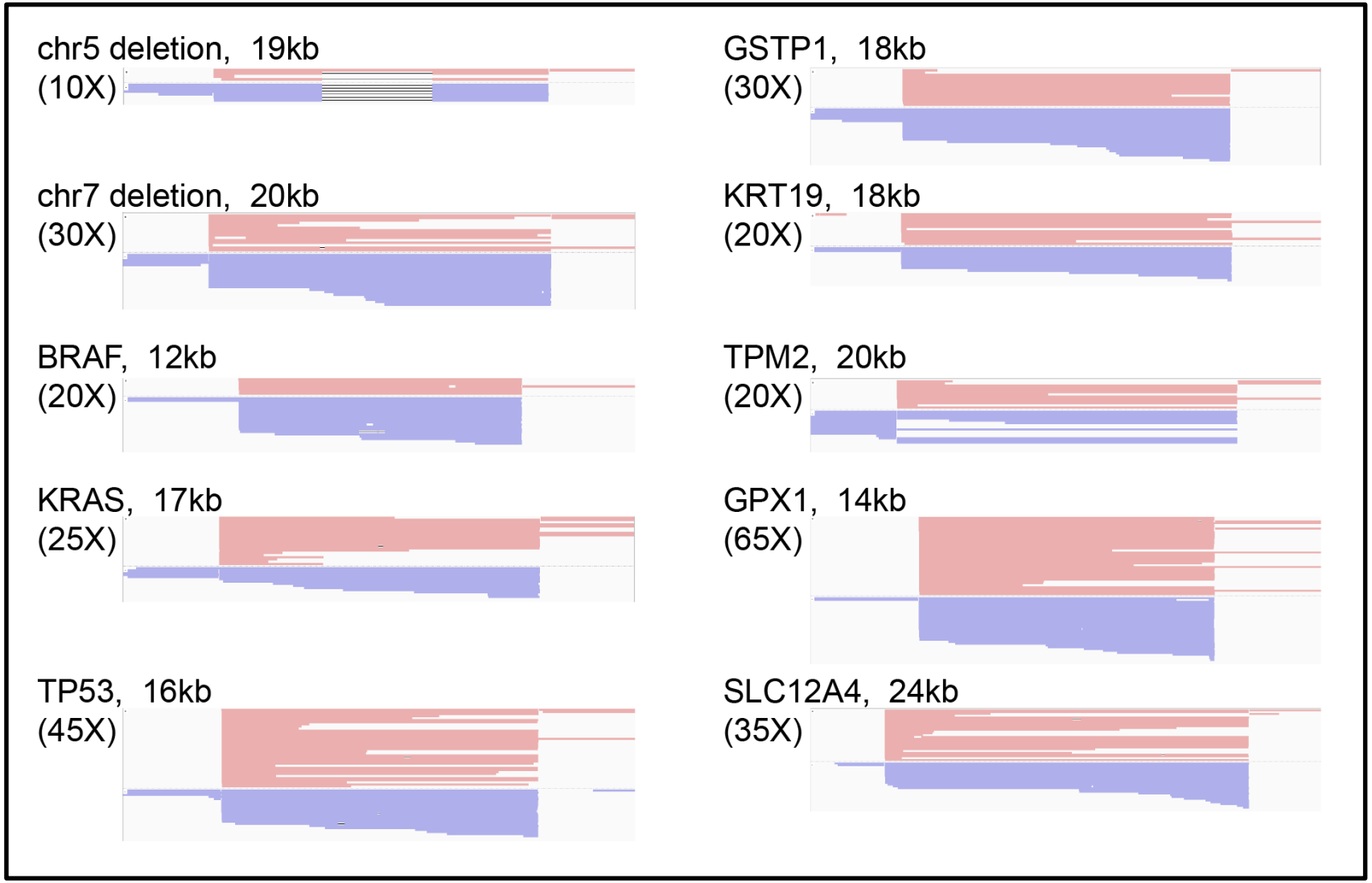
- Yield from
- 3ug GM12878 gDNA
- MinION Flow cell



Gilpatrick et al bioRxiv (2019)

Using a panel of guideRNAs

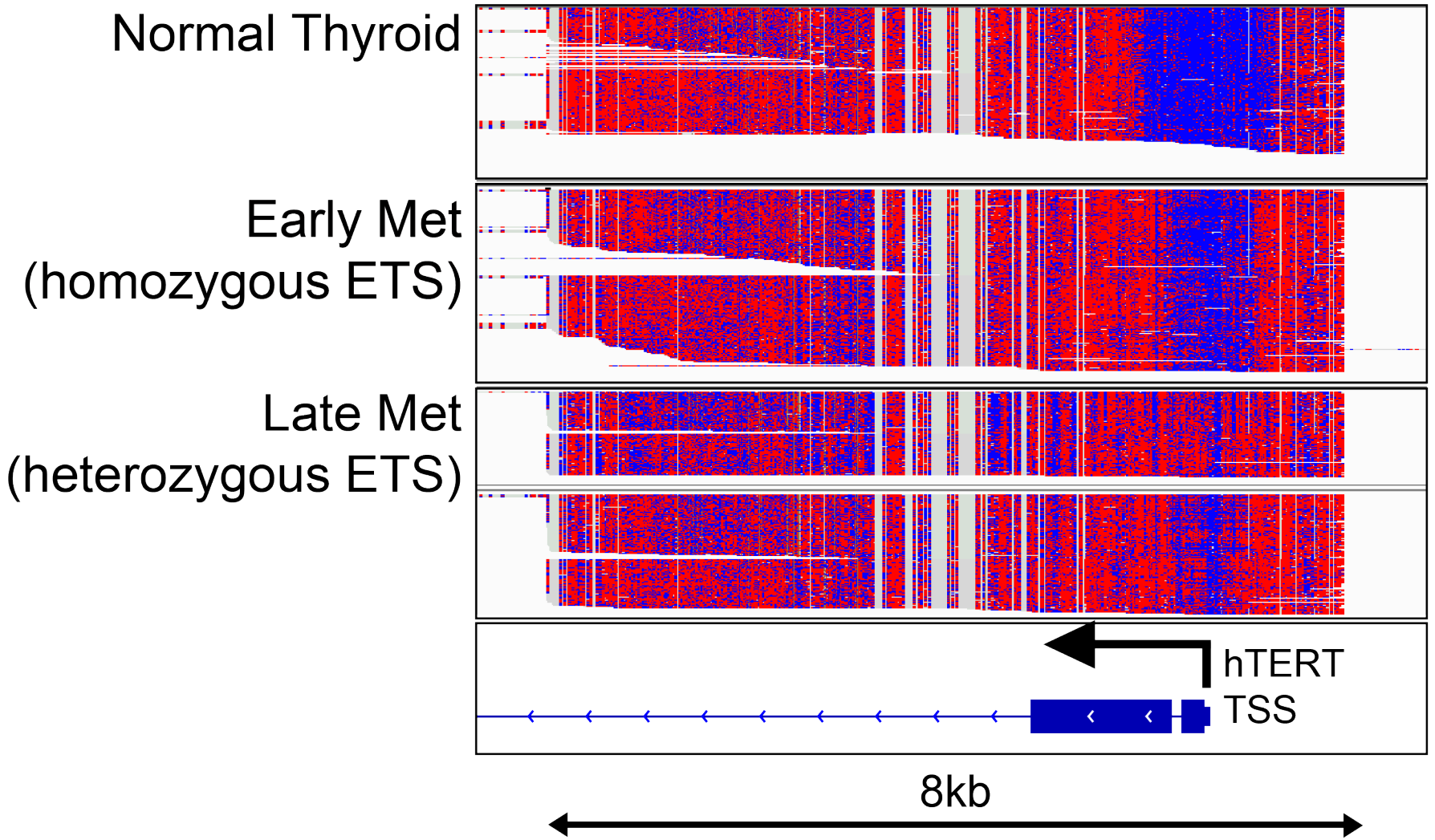
- Yield from
- 3ug GM12878 gDNA
- Flongle Flow cell



Gilpatrick et al bioRxiv (2019)

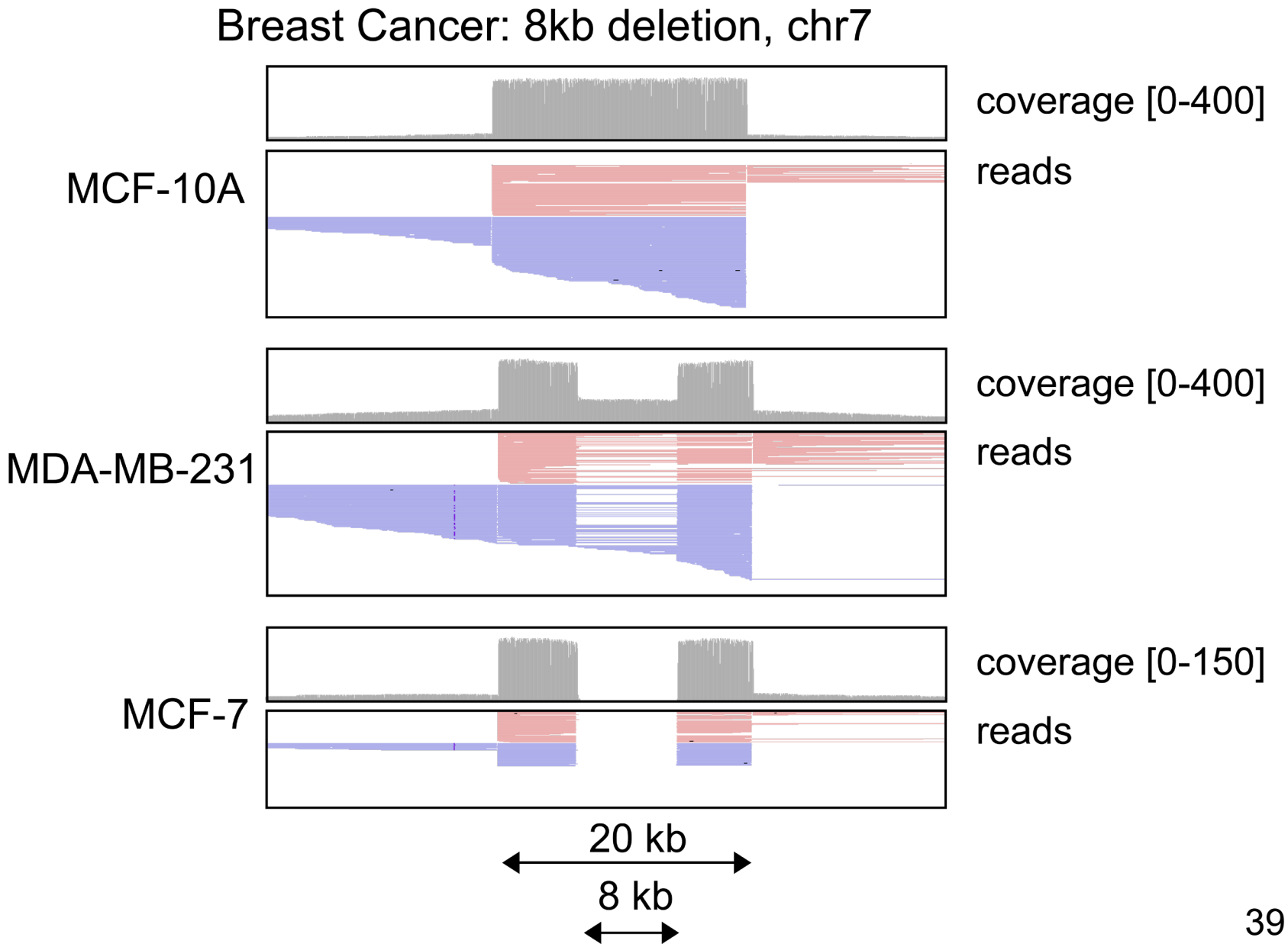
Enrichment of hTERT region

- We observe an “erosion” of the unmethylated (blue) CpG island in the promoter of hTERT in progressive cancer samples
- In the late metastasis a mutation in the ETS binding site of the promoter occurs in one of the alleles
- The mutant allele appears to have a more unmethylated island than the WT allele



Structural Variants in Cancer

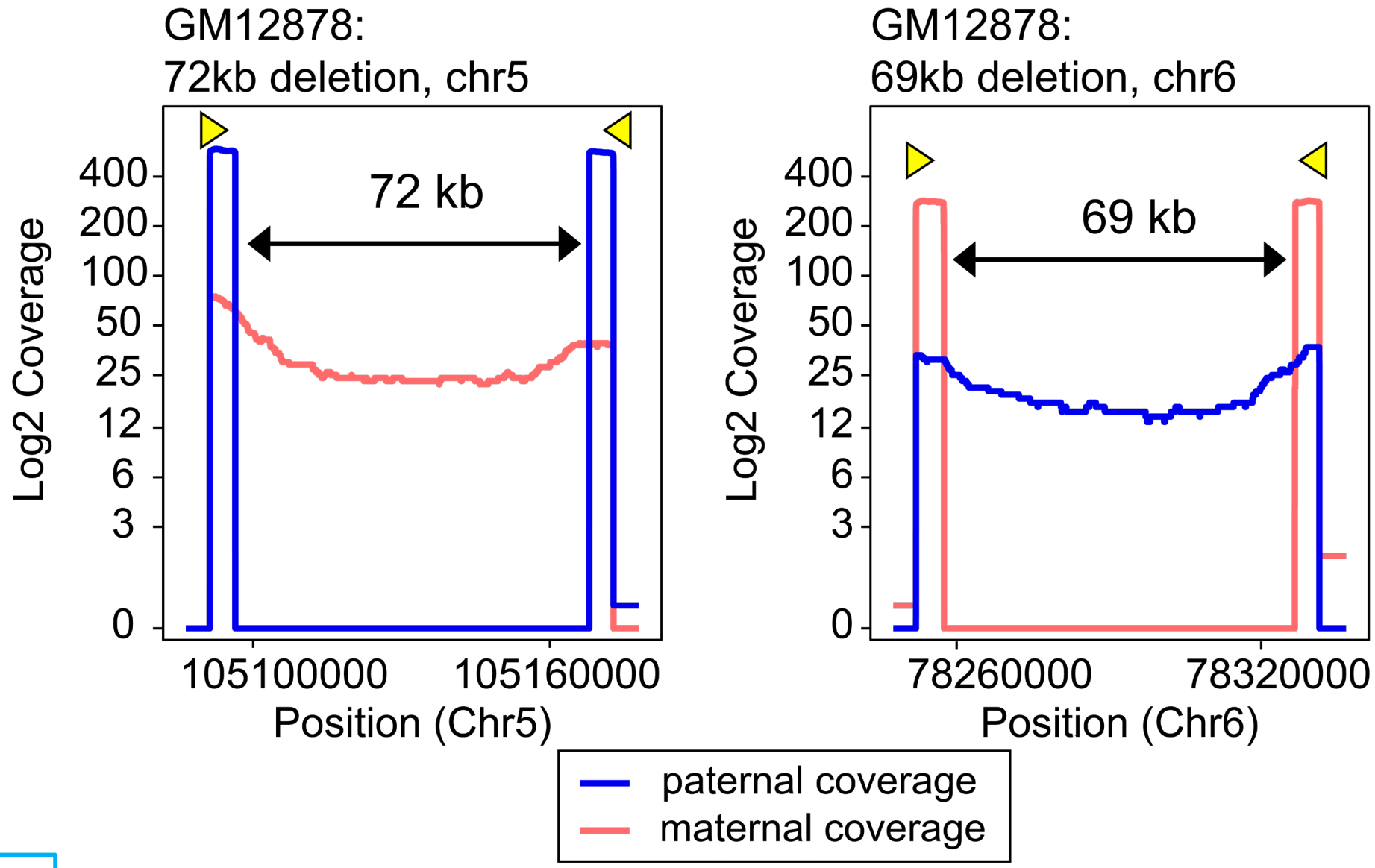
- Structural variants (SV), large insertions, deletions or translocations in the genome, are hard to detect with short-read sequencing
- Nanopore sequencing can map them well, and with targeted sequencing we can observe these



Gilpatrick et al bioRxiv (2019)

Structural Variation Detection

- Deleted allele has >200X coverage, but intact allele has 1/10 as much coverage
- Bias likely due to length of reads input into enrichment



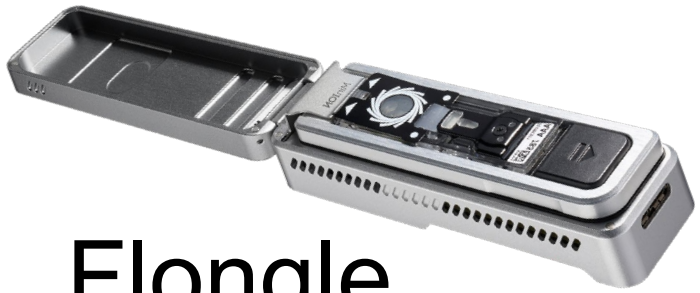
Gilpatrick et al bioRxiv (2019)

Single Nucleotide Variants

176 known SNVs exist in in span of 140kb in GM12878

MinION

Avg Cov : 100X



Flongle

Avg Cov : 30X

MinION		TP	Sensitivity	FP	PPV
default variant calls	SAMTOOLS	170	0.97	12	0.93
	NANOPOLISH	169	0.96	17	0.91
dual-strand filter	SAMTOOLS	142	0.81	3	0.98
	NANOPOLISH	156	0.89	1	0.99

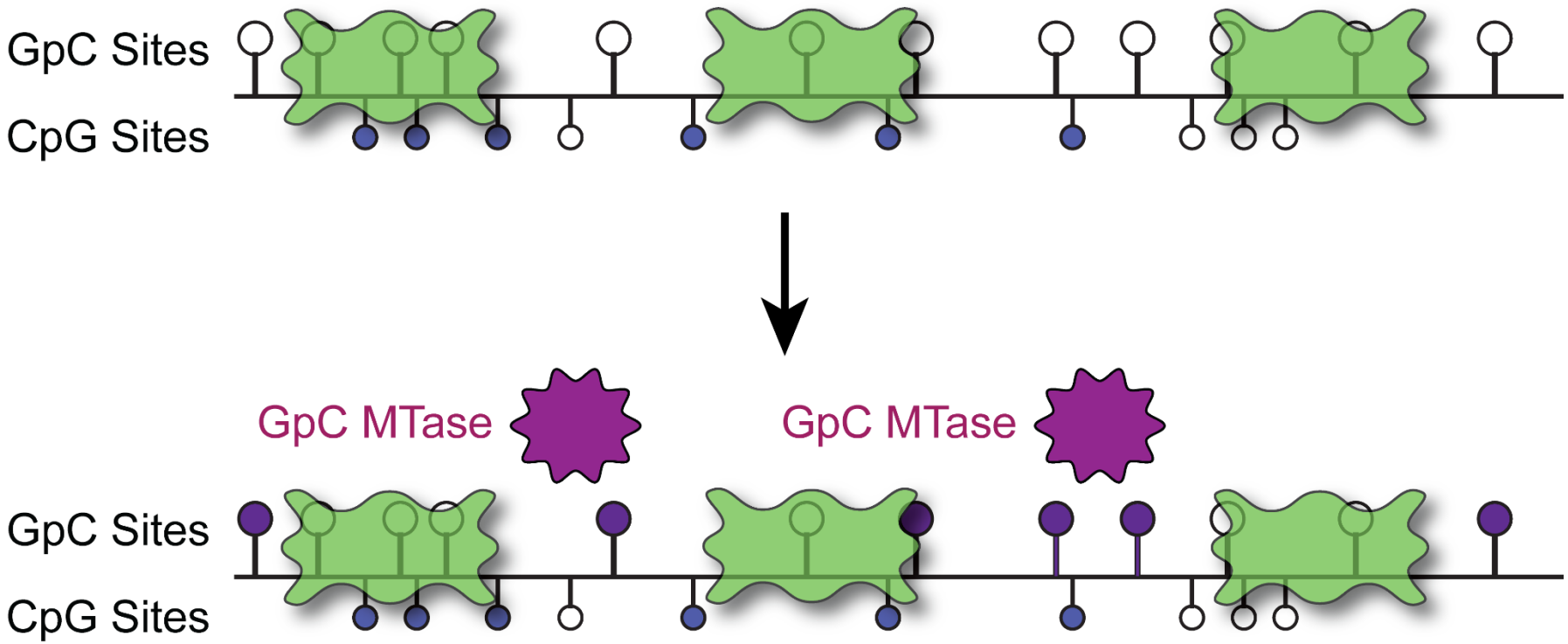
Flongle		TP	Sensitivity	FP	PPV
default variant calls	SAMTOOLS	138	0.78	10	0.93
	NANOPOLISH	160	0.91	20	0.89
dual-strand filter	SAMTOOLS	61	0.35	0	1.00
	NANOPOLISH	100	0.57	1	0.99

Gilpatrick et al bioRxiv (2019)

NanoNOMe: Chromatin Accessibility with Nanopore

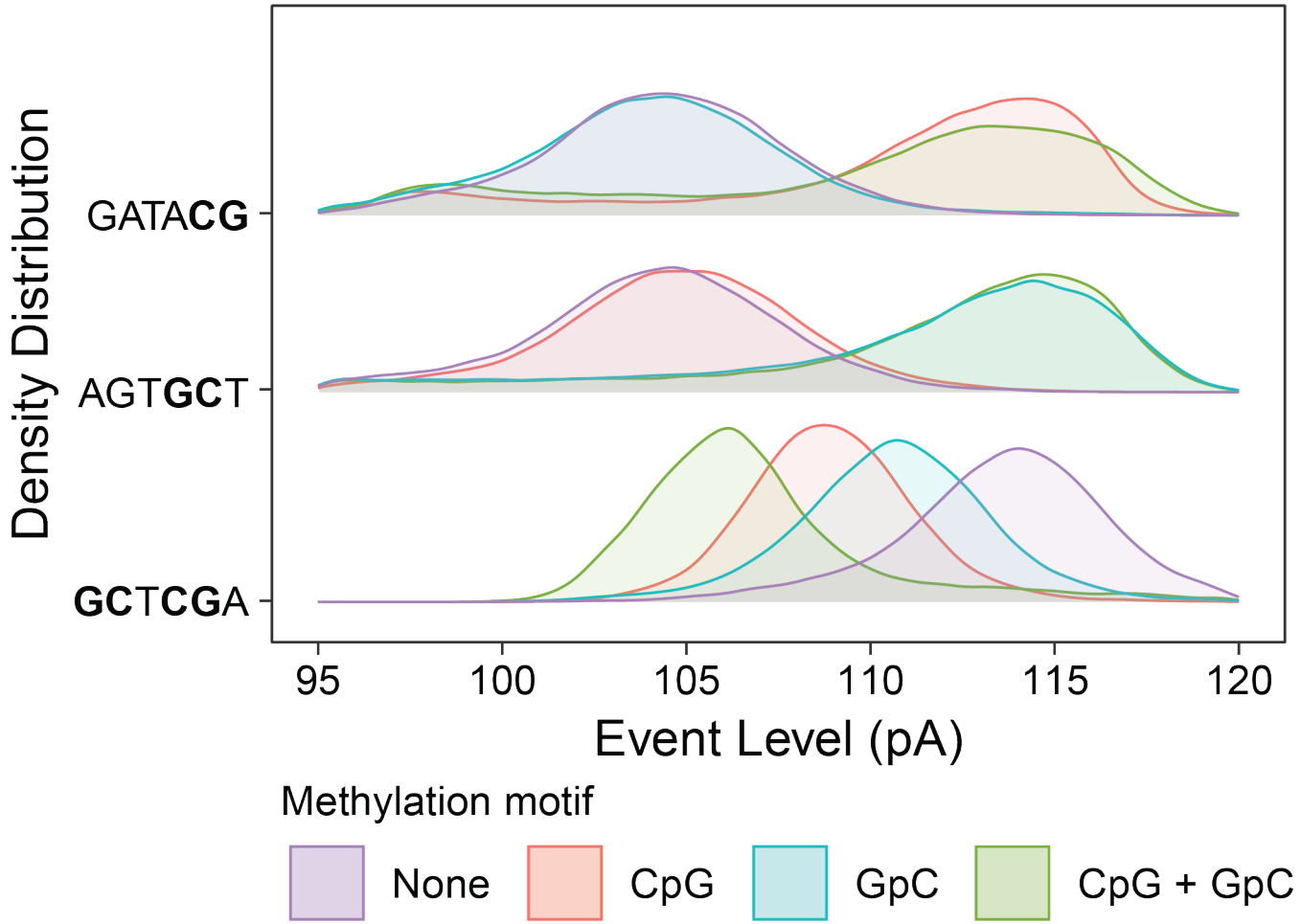
- NOMe-seq : Nucleosome Ocupancy and Methylome sequencing (Kelly et. al. *Genome Res.* 2012)

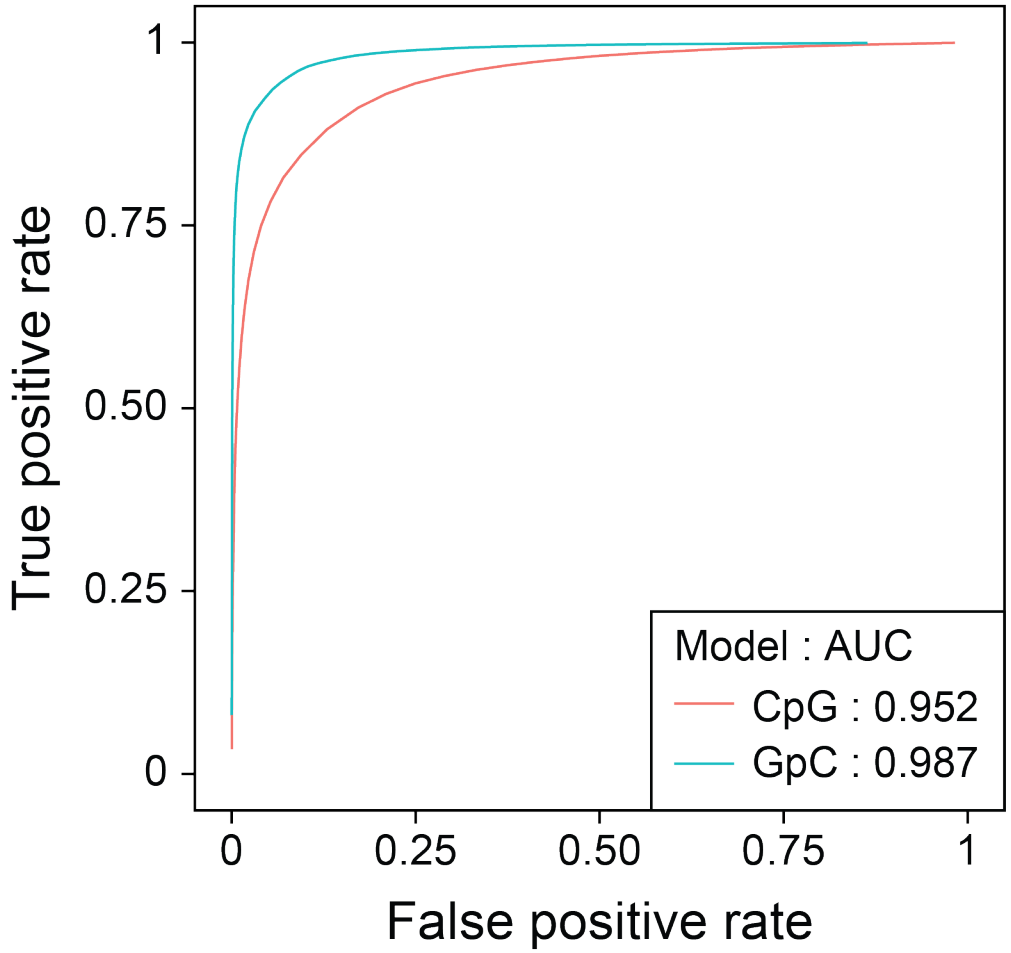
Simultaneously measures DNA methylation (CpG) and nucleosome occupancy (GpC)



Lee et al. bioRxiv 2018

Nanonome Signal

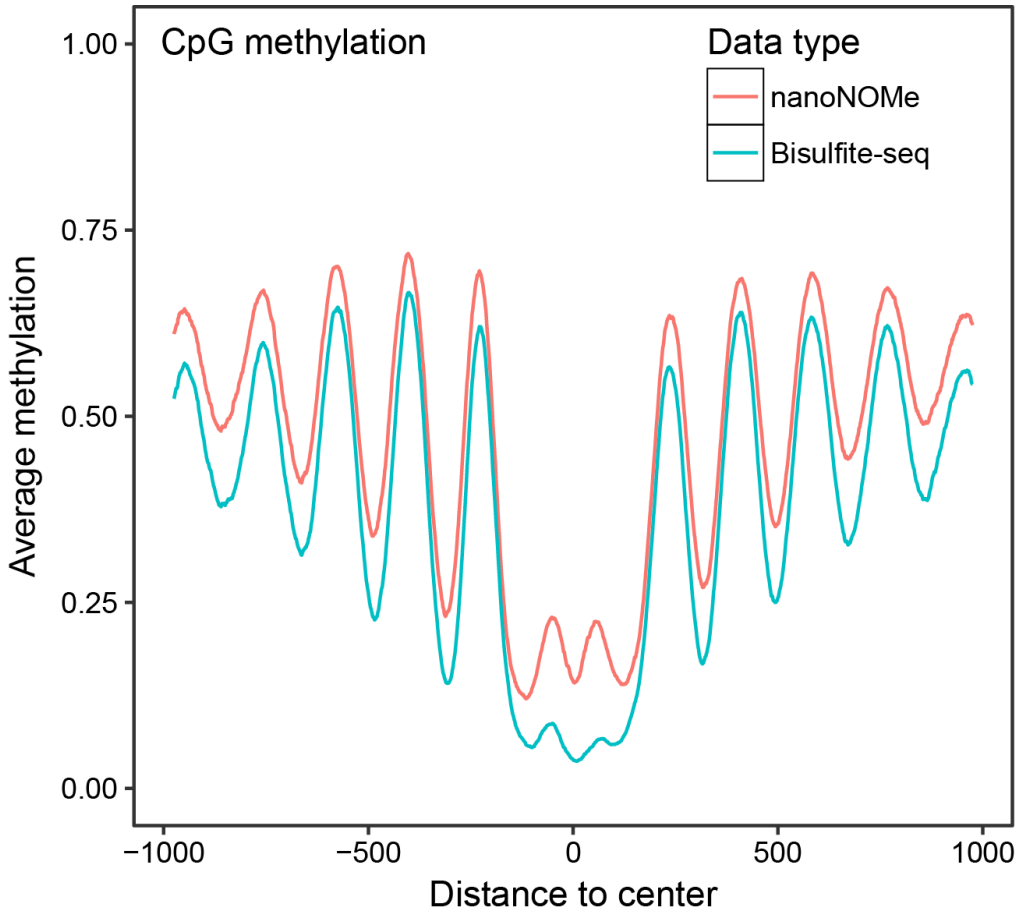


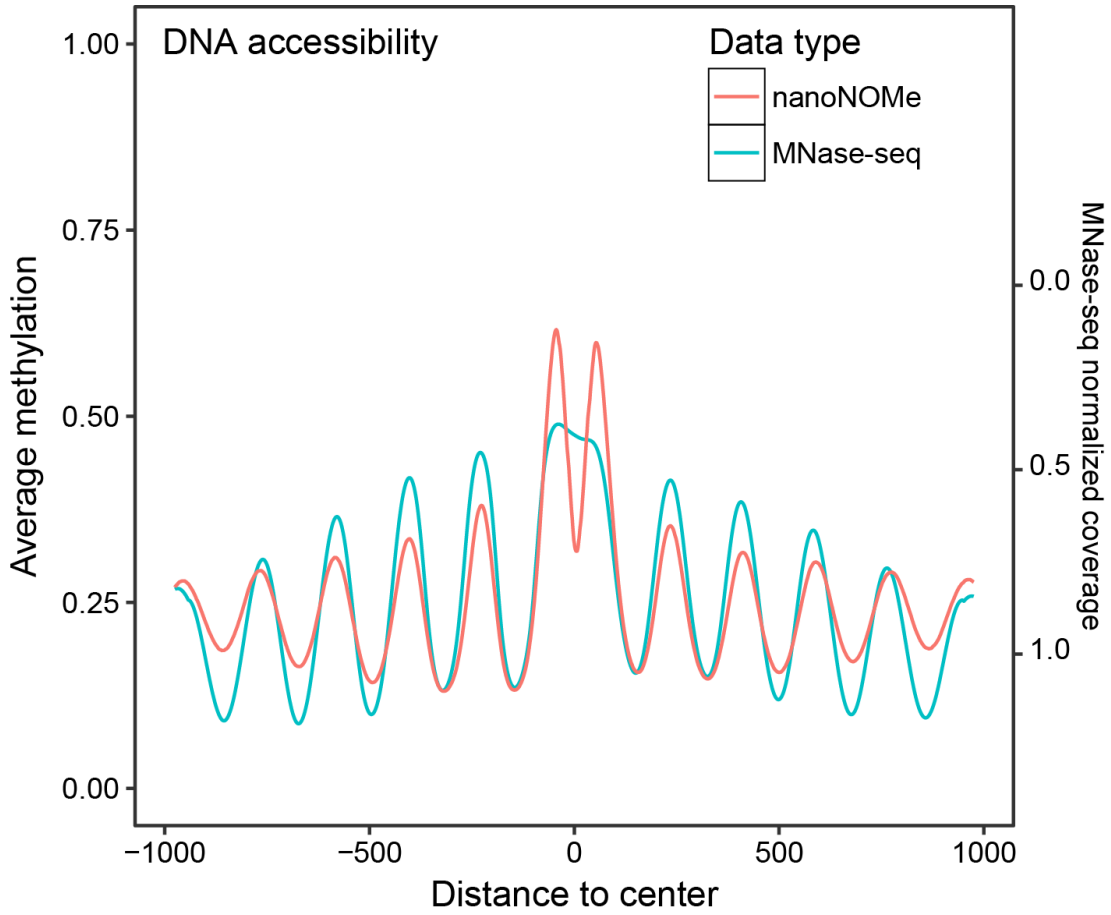


Lee et al. bioRxiv 2018

- 95% of CpGs as methylated in the 72% of all possible CpG k-mers
- 97% of GpCs as methylated in 89% of all possible GpC k-mers

NanoNOMe – DNAse Hypersensitive



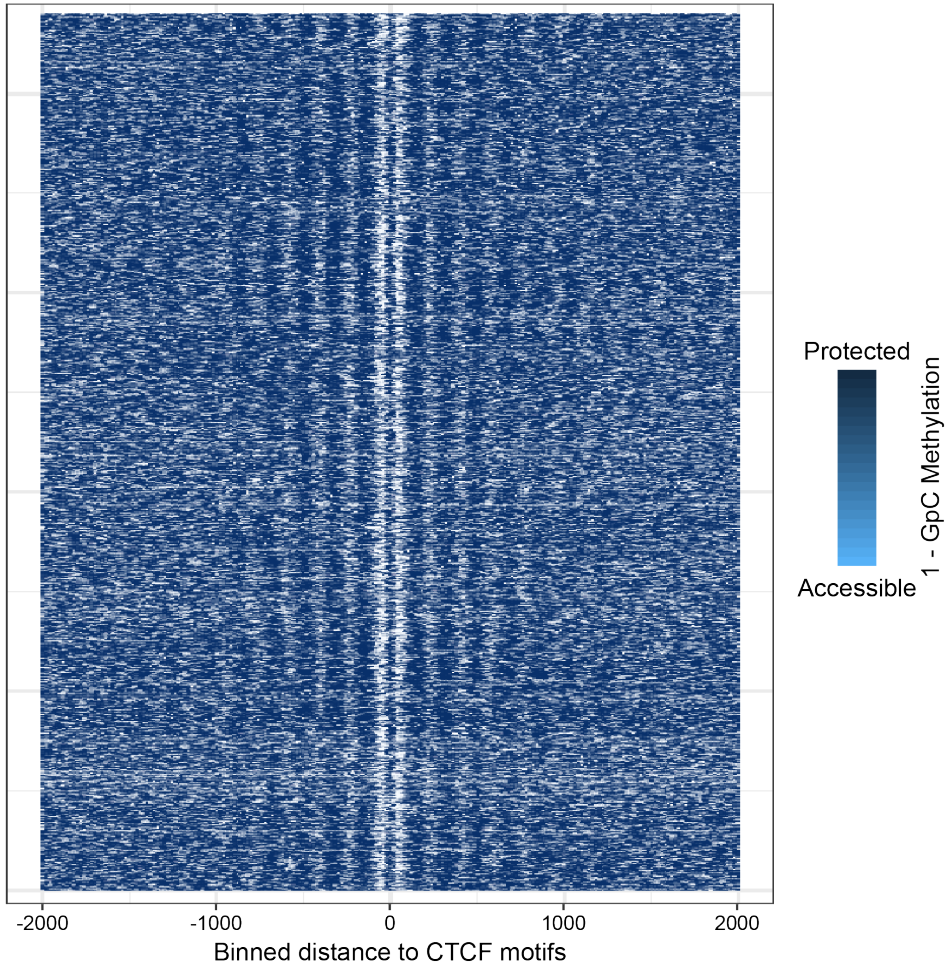


nanoNOMe signal near DNAse-seq peaks validates the methodology

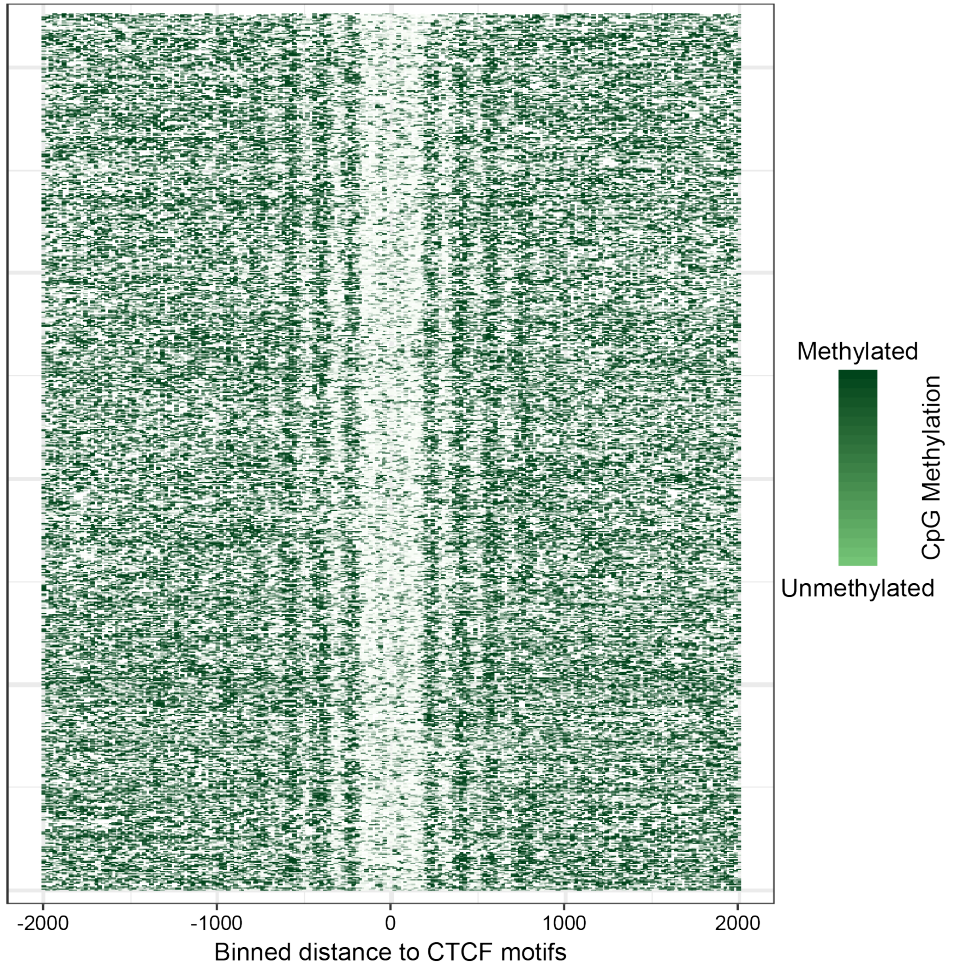
Lee et al. bioRxiv 2018

NanoNOMe: Aggregate CTCF binding sites

Chromatin Protection (1-GpC)



Endogenous Methylation (CpG)

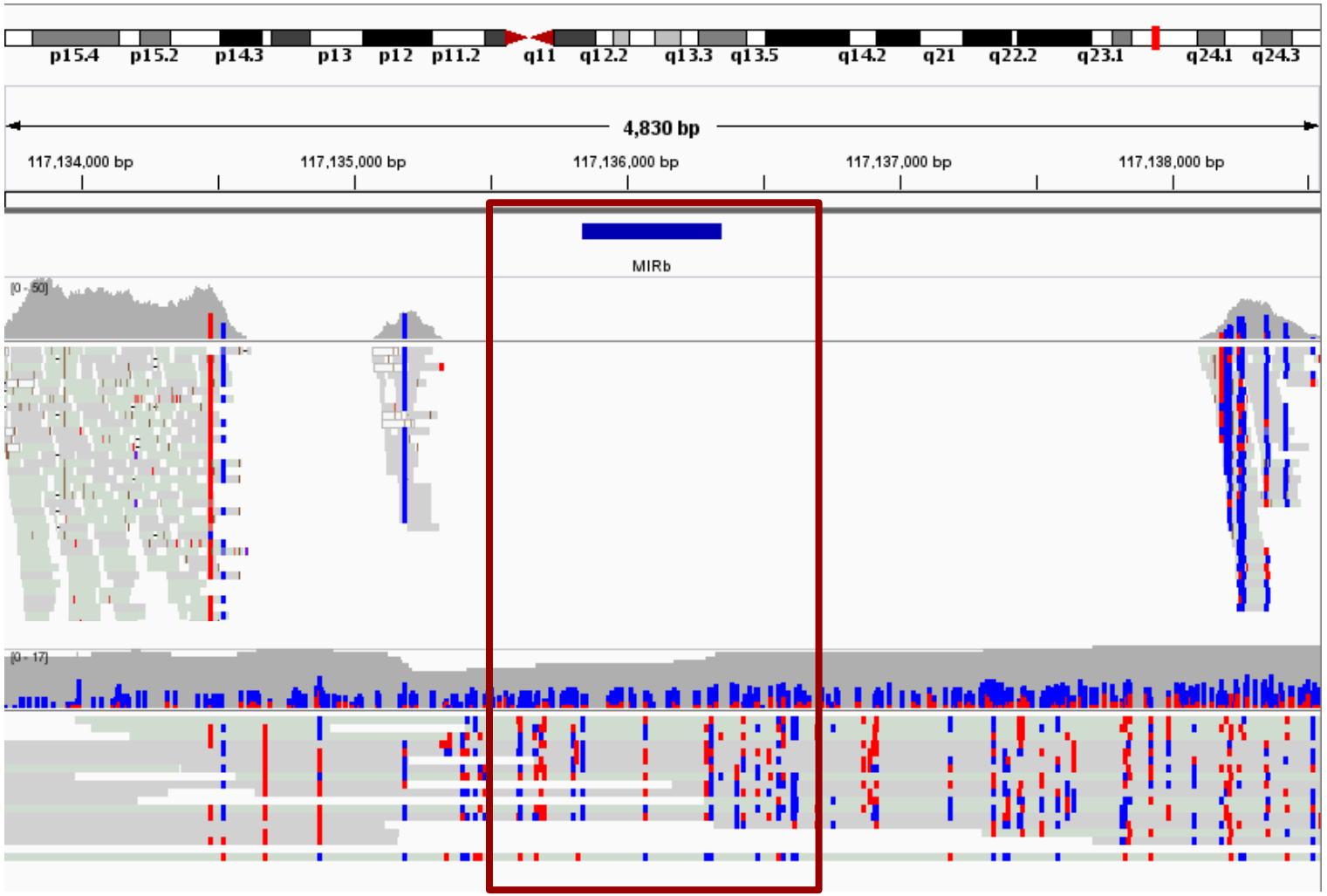


Lee et al. bioRxiv 2018

Methylation in Repetitive Regions

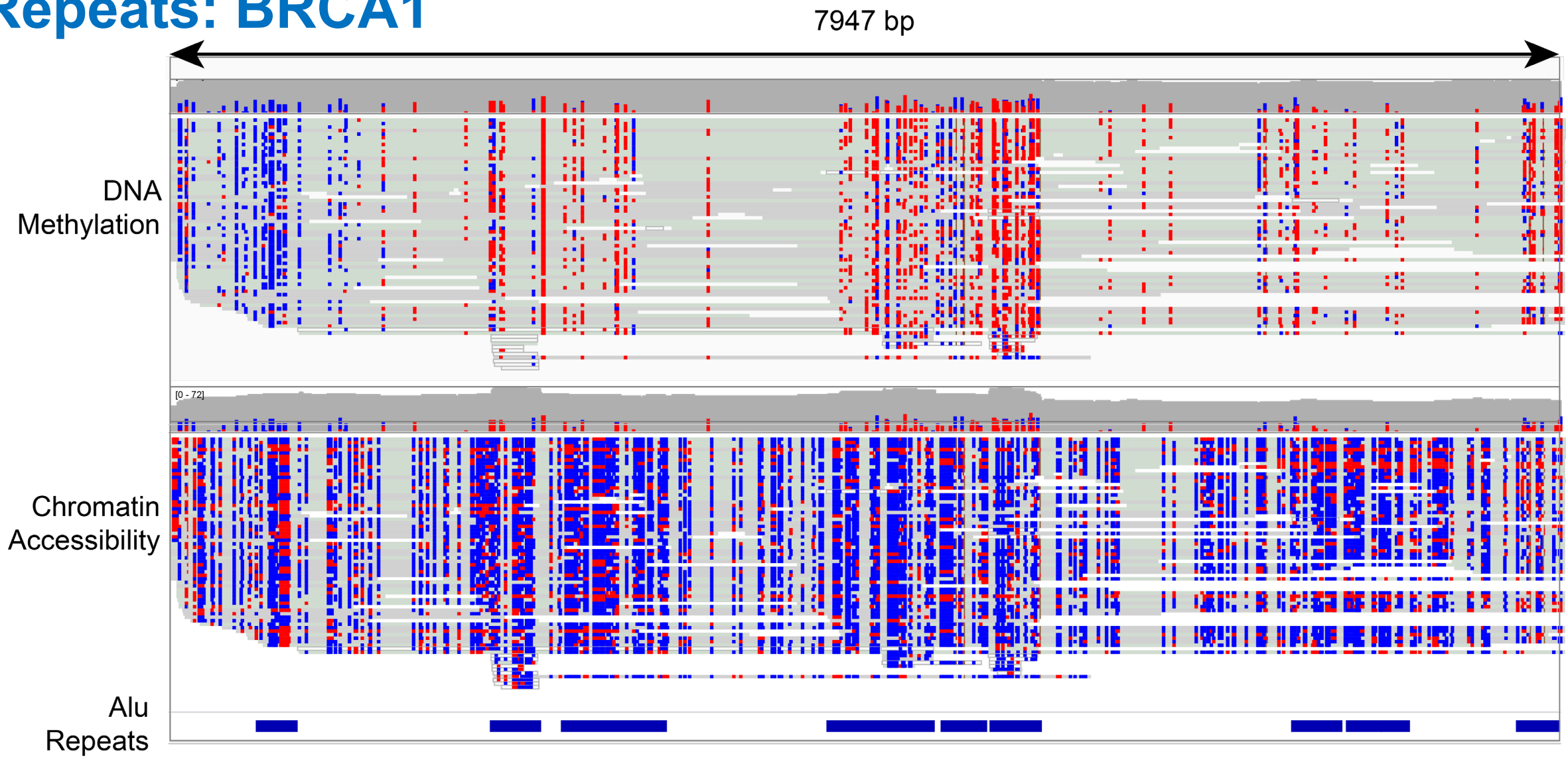
Bisulfite Sequencing (Illumina)

nanopolish methcall (Nanopore)



Regions unmappable by NGS are mappable with long reads

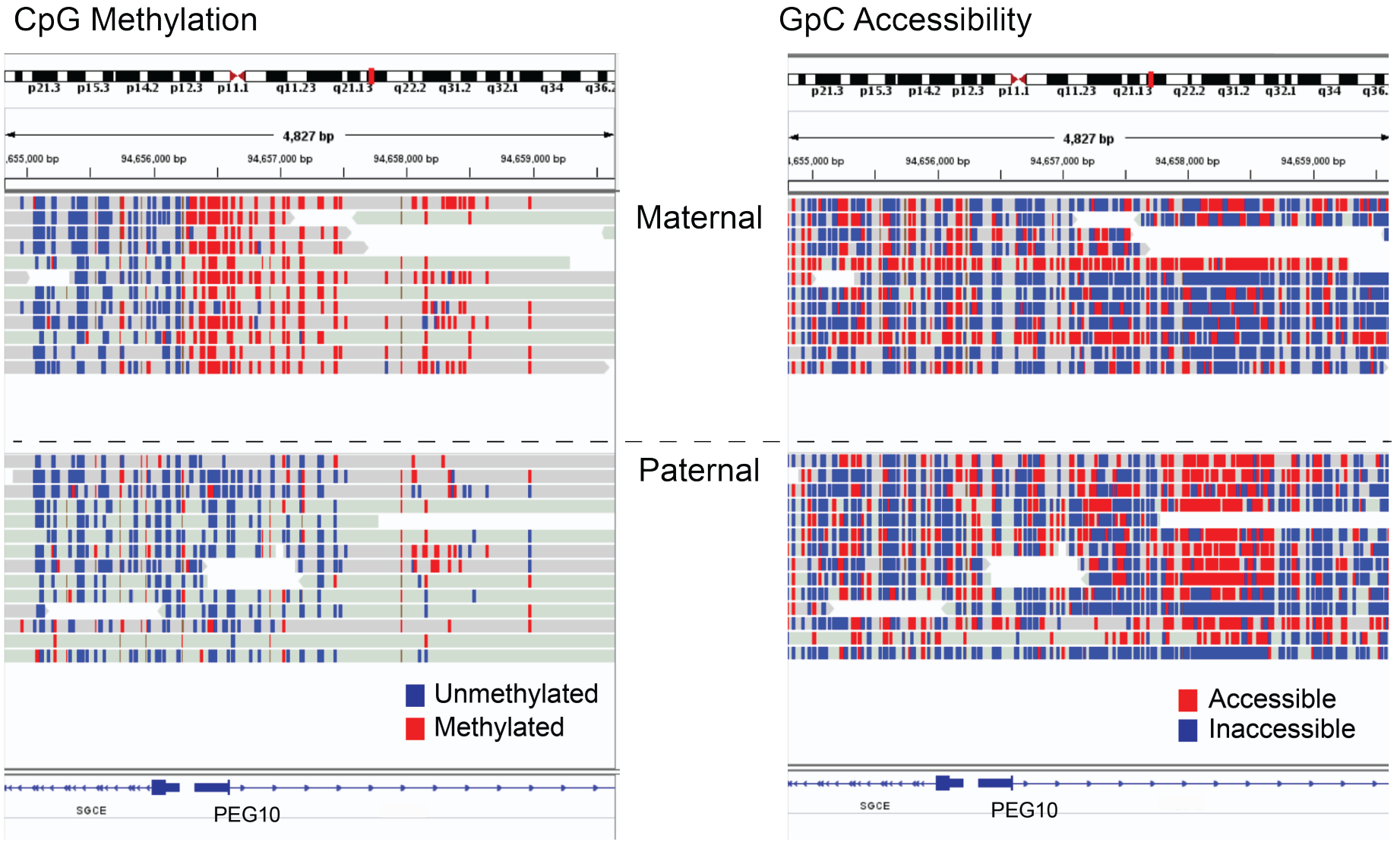
Repeats: BRCA1



Reference genome doesn't have many of these repeats properly – for BRCA1 region we aligned our reads against a custom GM12878 genome assembly (Jain et al)

Allele Specific Chromatin and Methylation

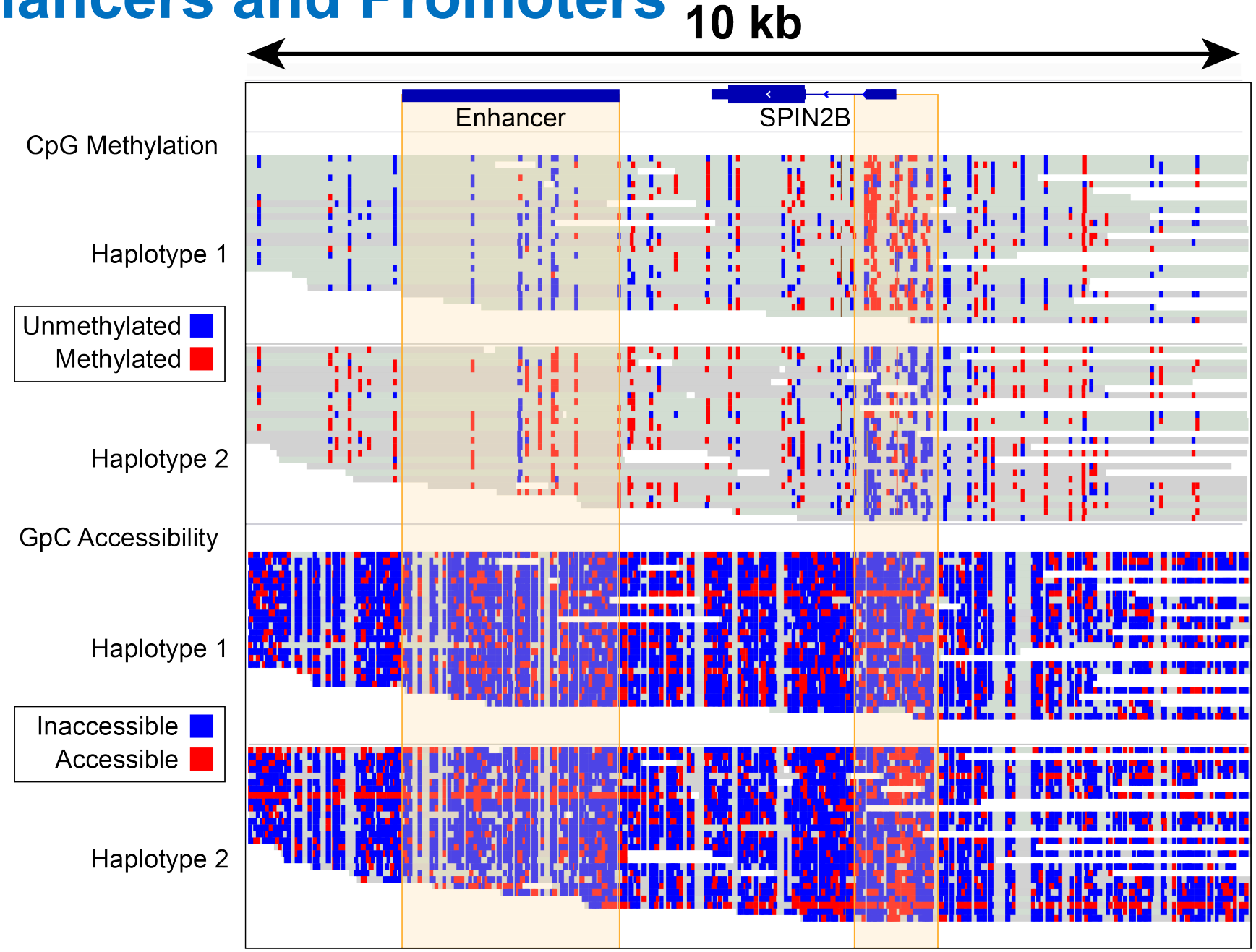
- Using long reads, we are likely to encounter a SNP
- This allows for phased methylation and chromatin data
- Near *PEG10* (imprinted gene):
  - Maternal copy is methylated and inaccessible
  - Paternal copy is unmethylated and accessible



Lee et al. bioRxiv 2018

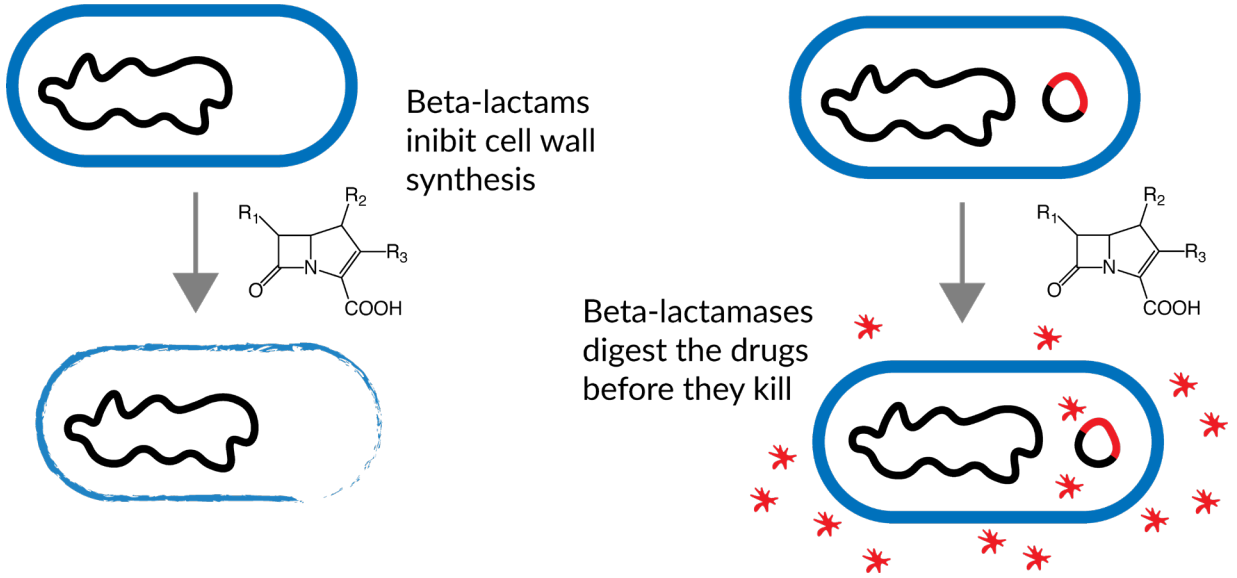
Coordinated Enhancers and Promoters

Using long reads, we can examine methylation and chromatin at some promoters and enhancers at the same time

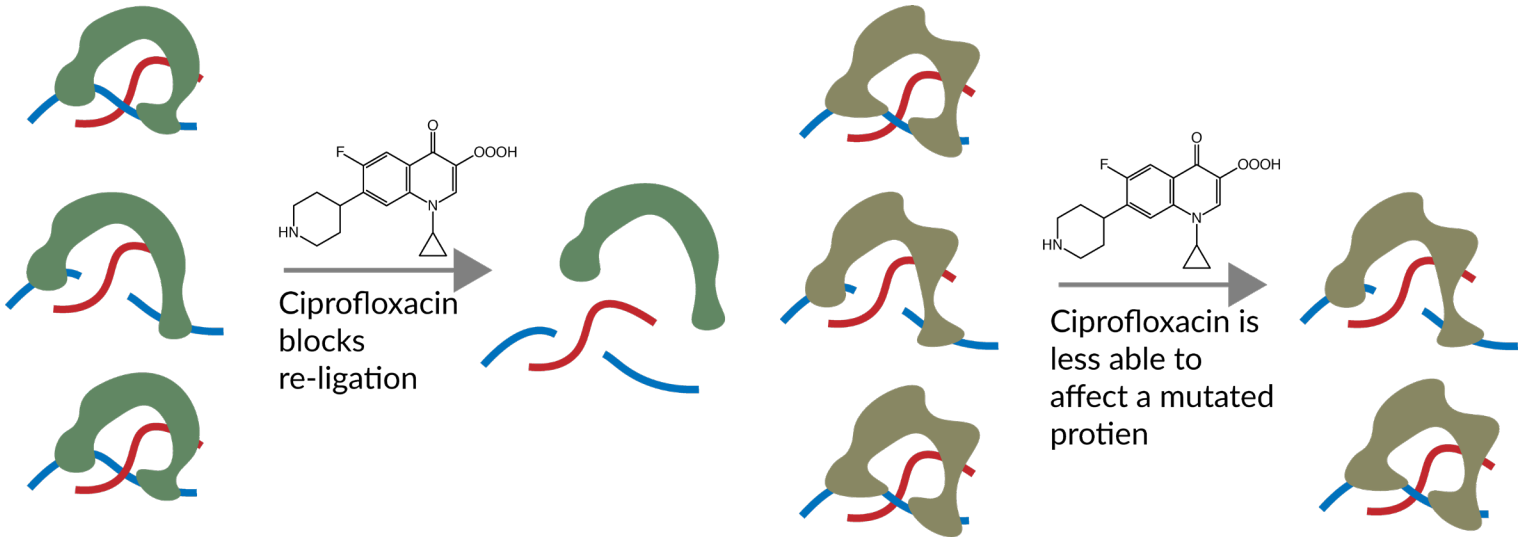


Lee et al. bioRxiv 2018

Antimicrobial Resistance



Resistance genes can be acquired via plasmids.



Mutations of otherwise benign genes can confer resistance.

(gyrA)

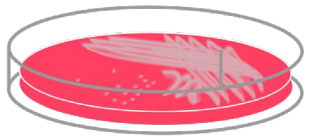
Aldred et al *Biochemistry* (Feb

Project Overview

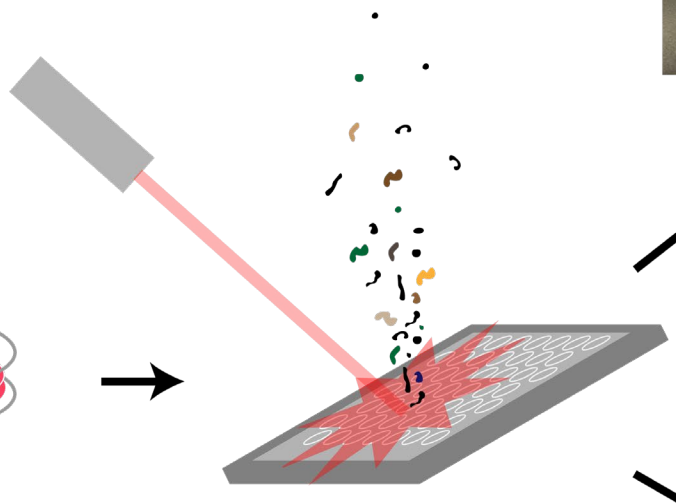
➔ Correlate resistance phenotypes with genomic features

Patients

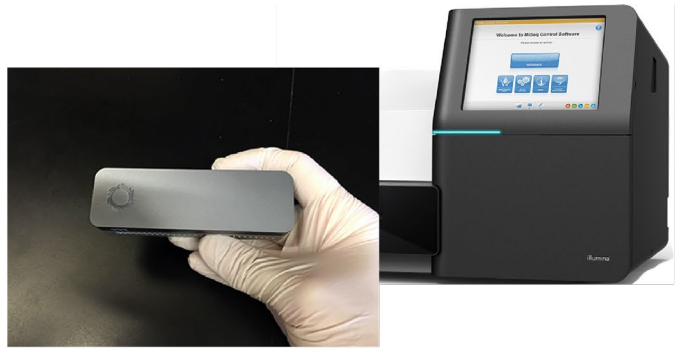
Rectal/
Perirectal
Swab



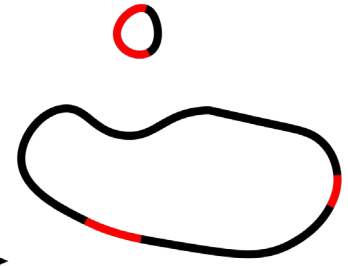
Culture



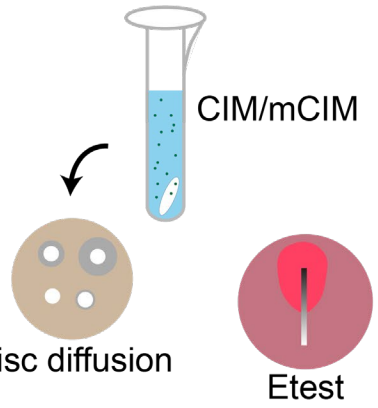
MALDI-TOF to
ID organisms



Nanopore (+ Illumina)
DNA Sequencing



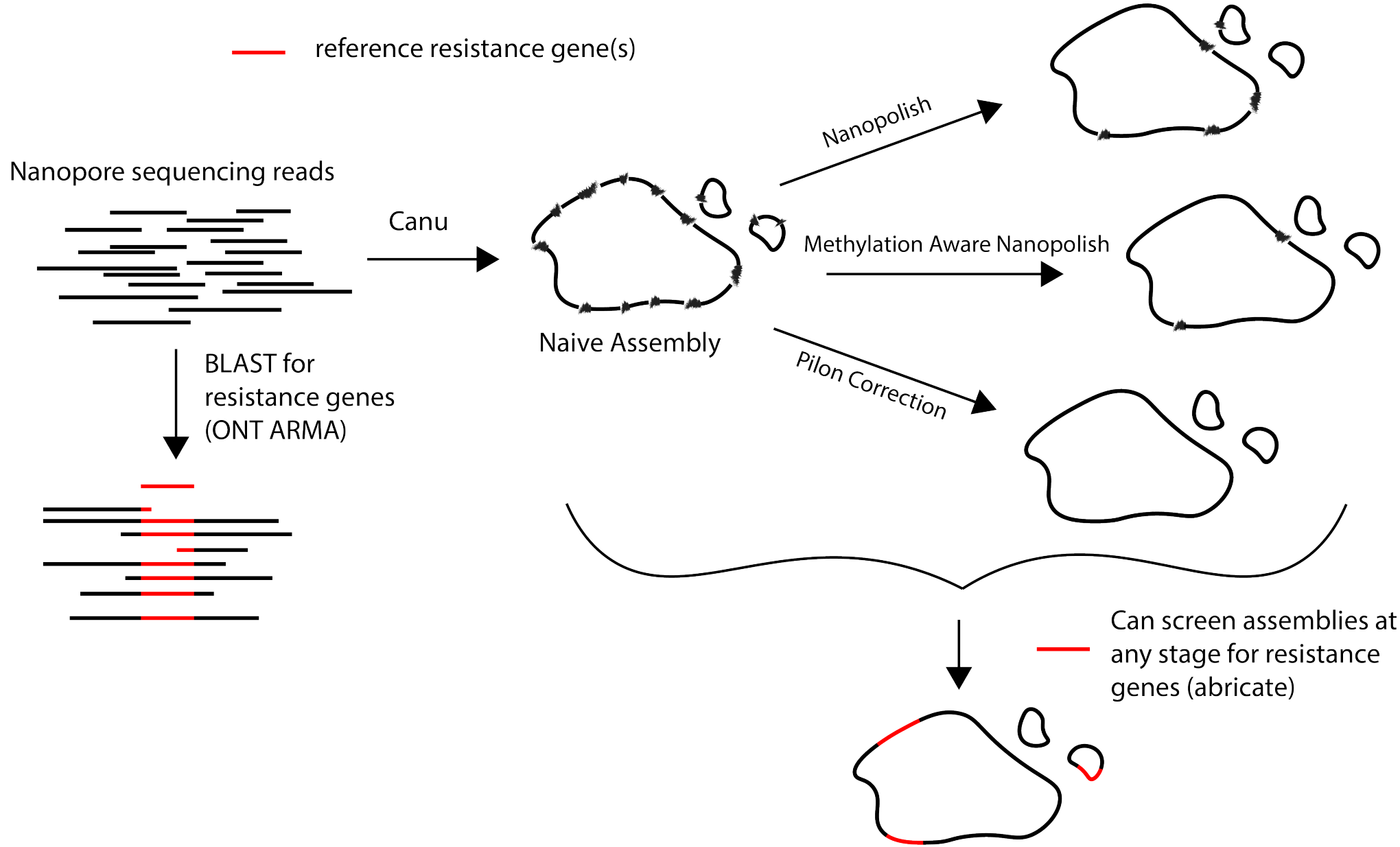
Assemble genome,
find resistance genes
and mutations



Standard of Care Tests

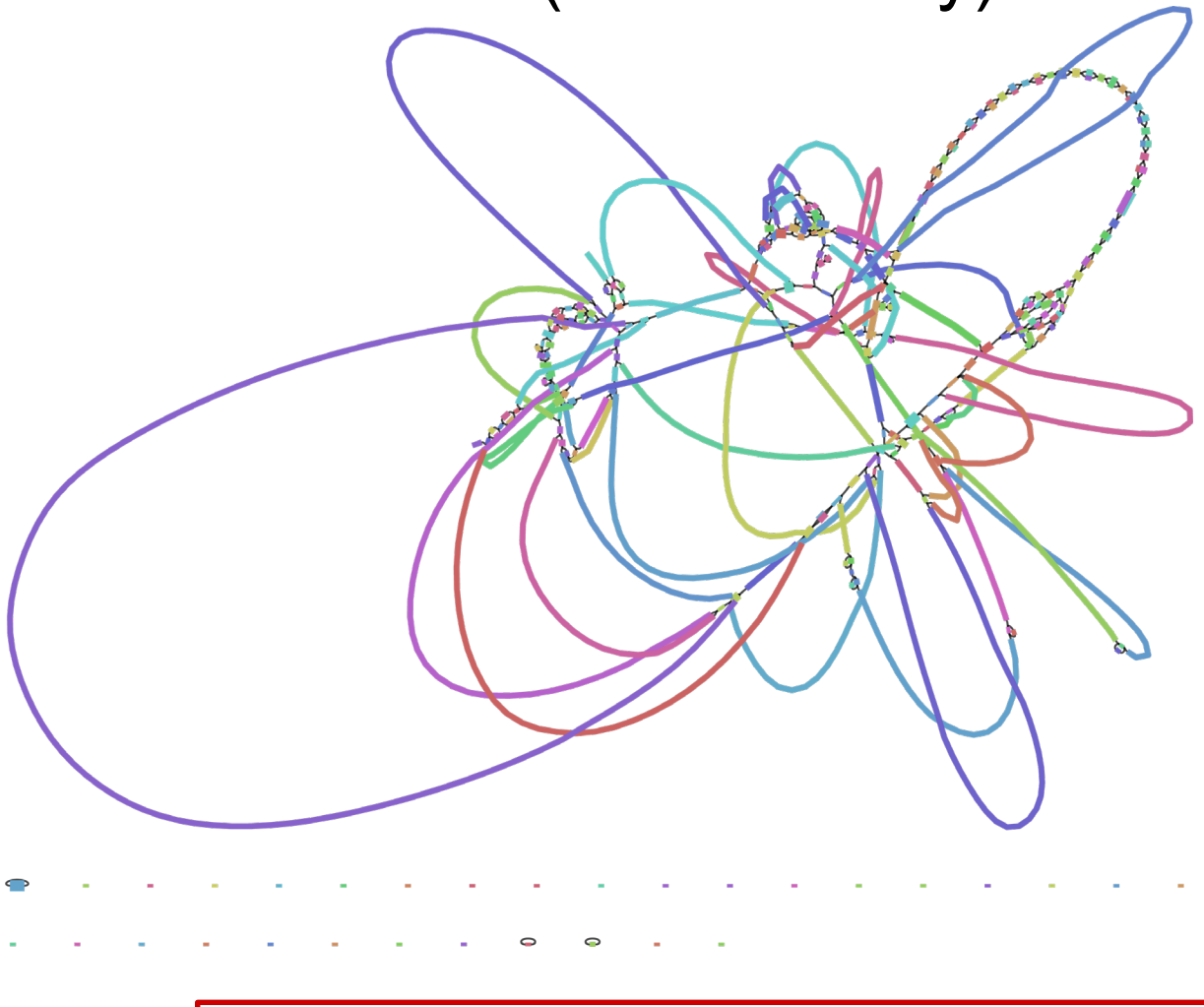
Antibiotic
Susceptibility
Profiles

The Pipeline so far

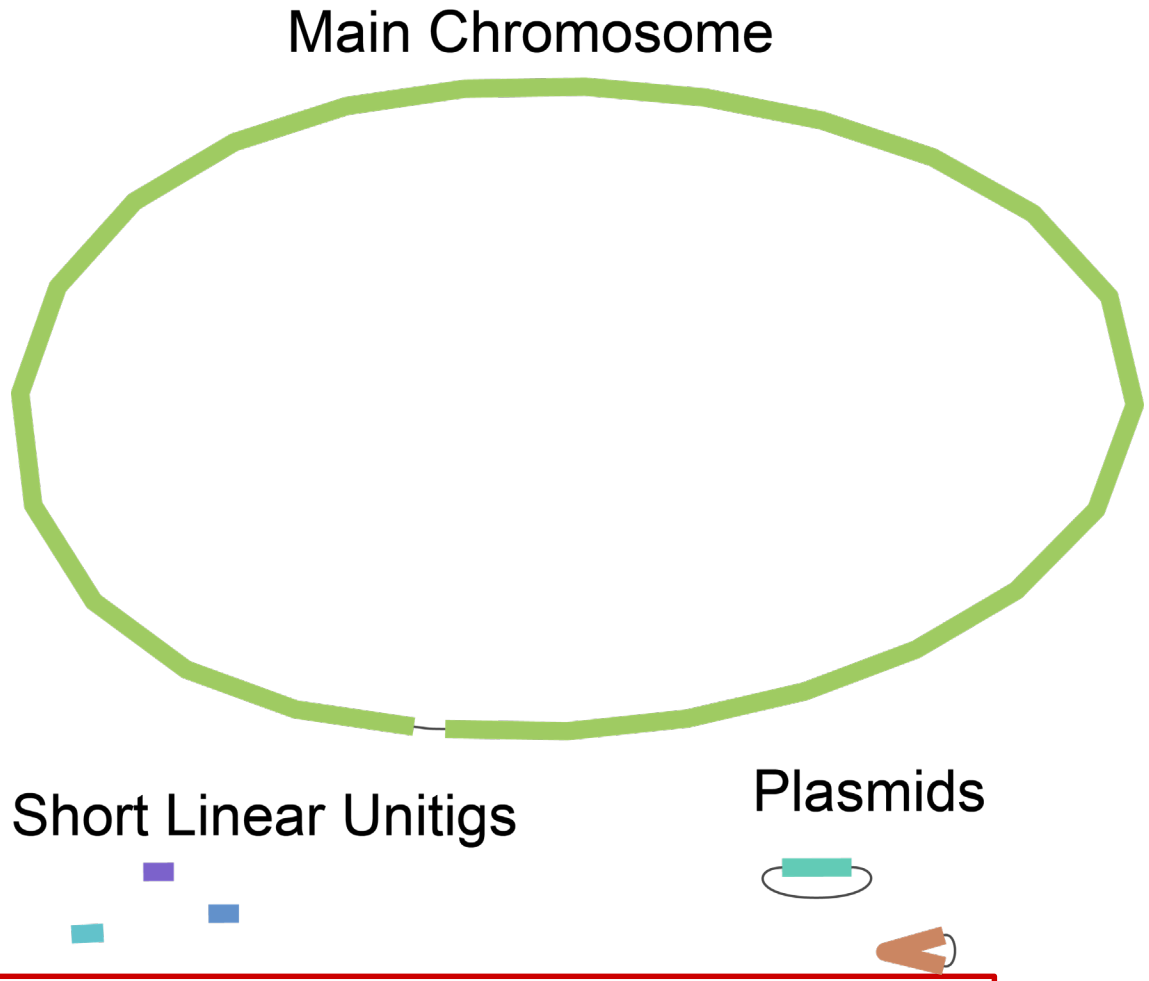


Assemblies

SPAdes (Illumina only)



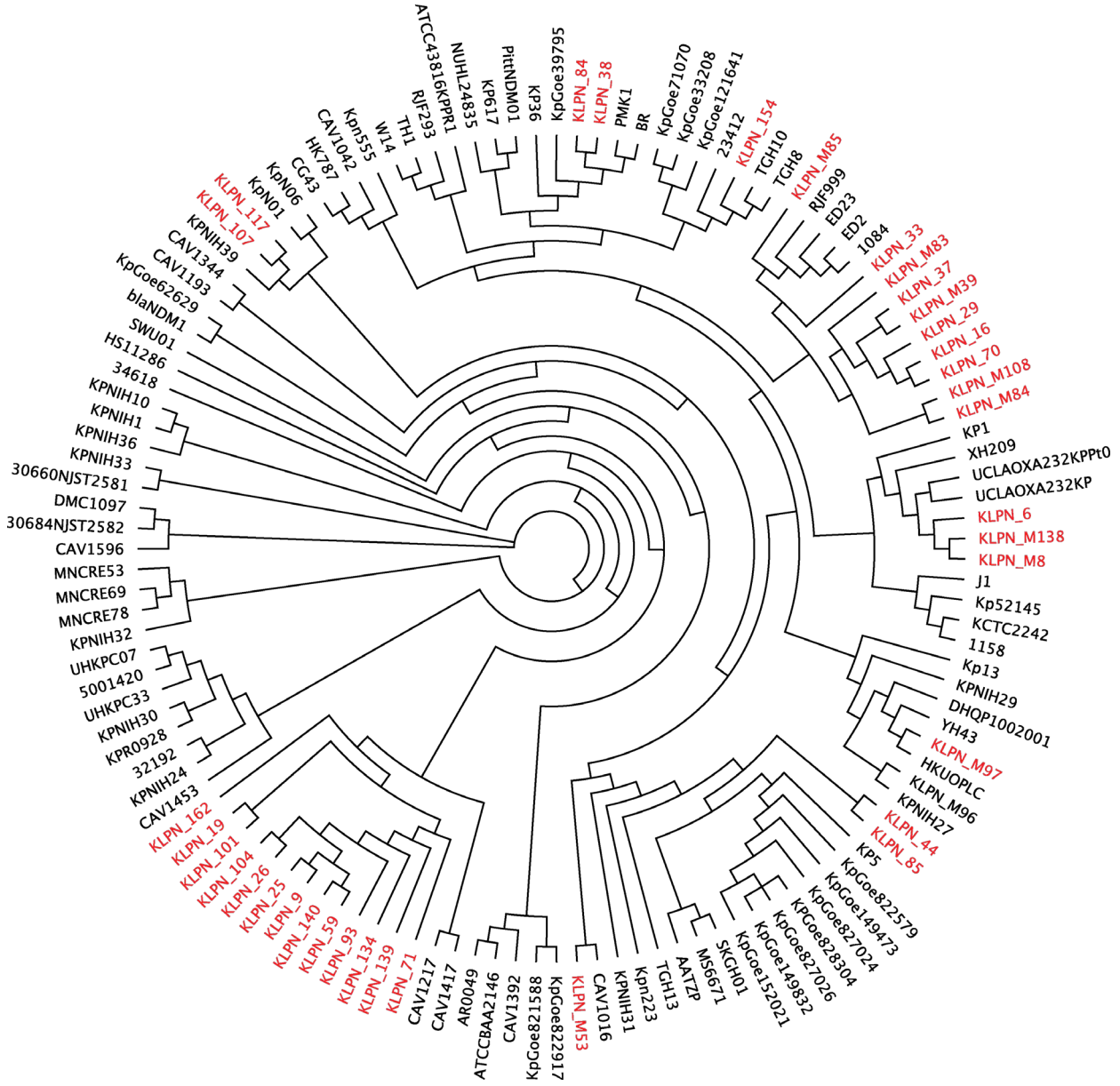
Canu (Nanopore only)



Long reads **really** help in getting complete assemblies – full single contig chromosomes and plasmids identified cleanly.

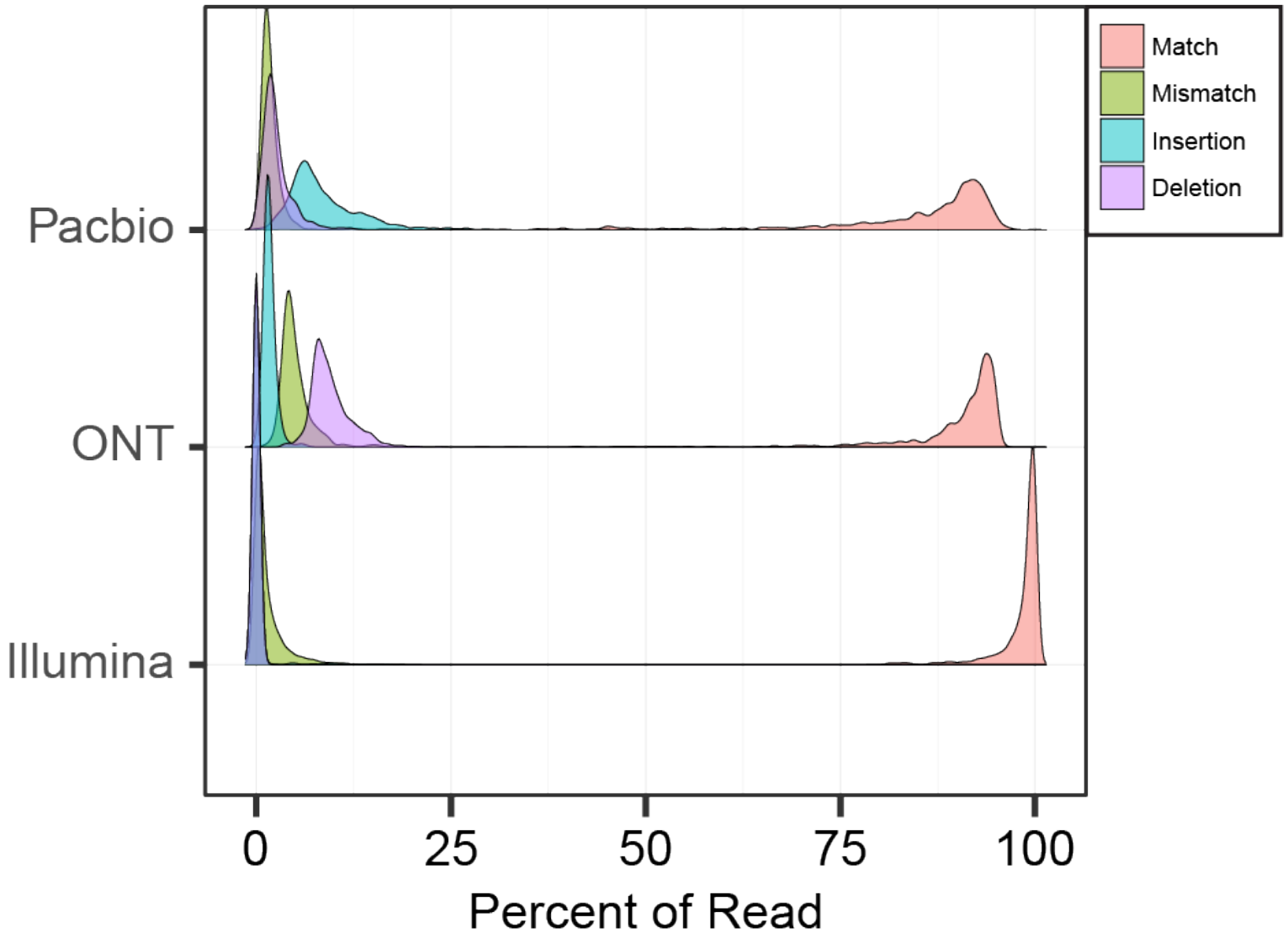
KLPN: Phylogeny in the context of NCBI genomes

- A comparison of our isolate assemblies to NCBI reference genomes for K. pneumo gave clear clustering with specific strains.
- Core whole-genome alignment with parsnp against complete *K. pneumo*



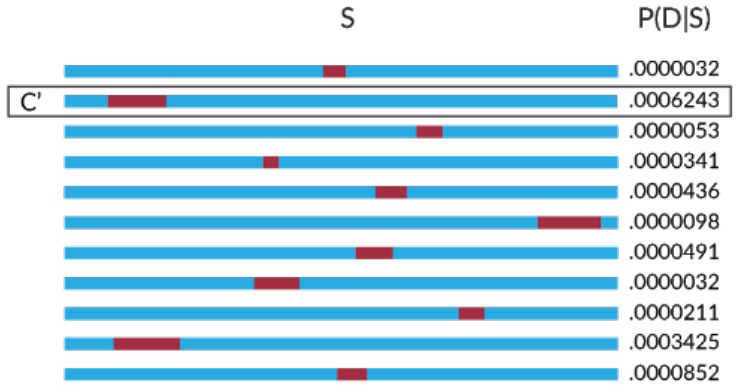
Alignment Error Comparison

- We used bowtie2 for Illumina and minimap2 for Pacbio and ONT alignment, then used samtools to compare to reference. For this comparison we used Illumina & Pacbio provided data compared to data from Nick Loman on *E. Coli*.
- Illumina reads which align do so nearly perfectly, with a per read median of 99.3% correct.
- PacBio reads which have an median of 89.2% of the read correct. The most frequent error type is insertions (7.45% median) with mismatches only 1.5% median % of read.
- ONT reads from v9.4 (Nick Loman's data) have a per read median of 92.4% correct, with deletions (9%) and mismatches (4.5%) both at a relatively high median per read.

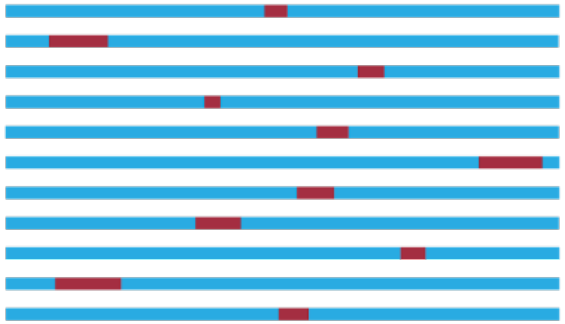


Nanopolish

Set the best S as the new C, and repeat

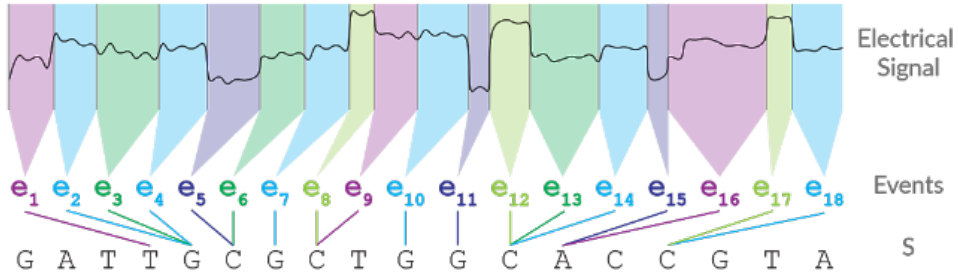


Based on non-matching stretches of sequence between the assembly and the reads, generate a list of candidate improvements to C, called S

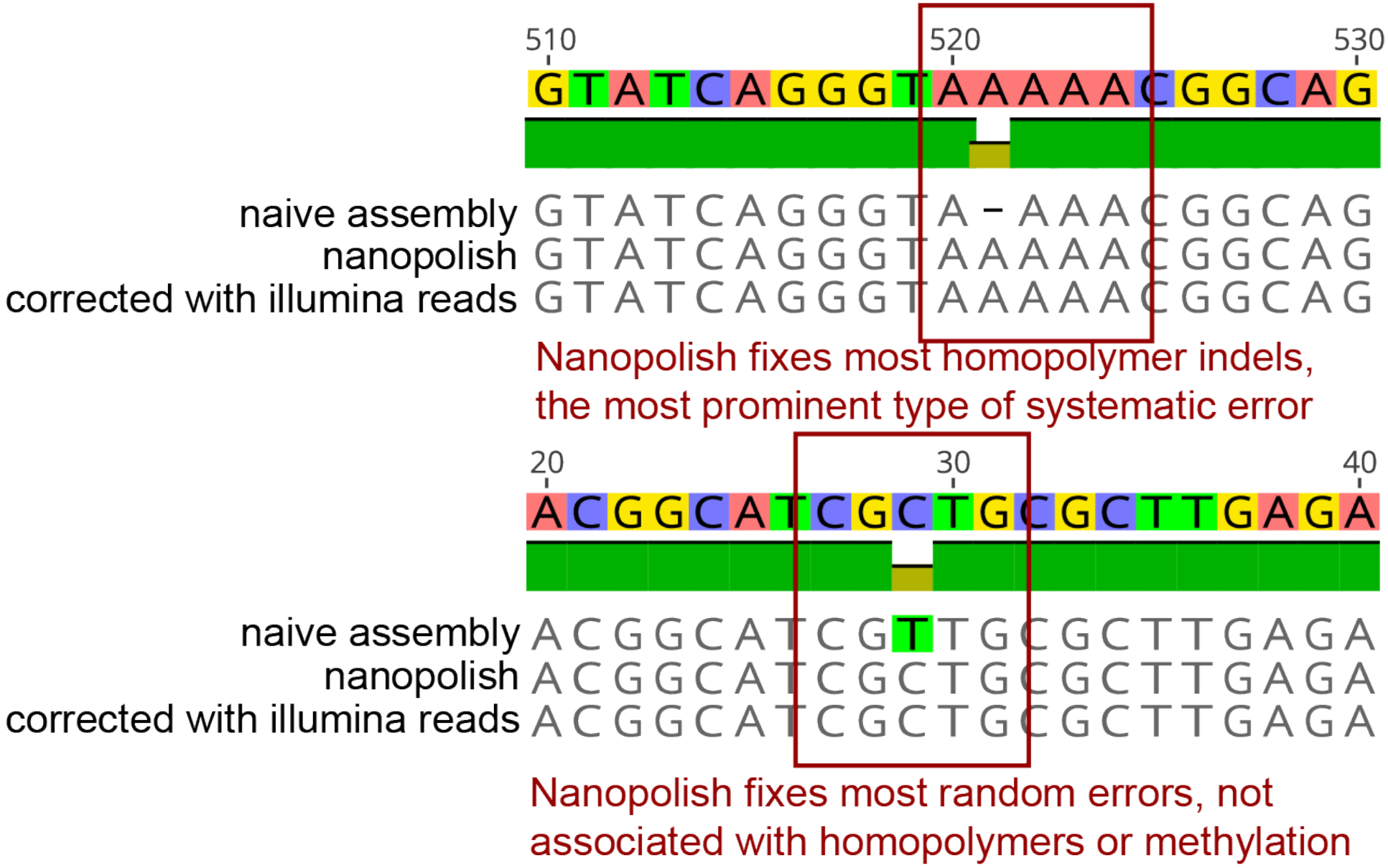


Use a Hidden Markov Model to align the electrical data from the reads to each S, and compute the probability of observing the event data given S, P(D|S)

Choose the S that maximizes the probability of observing the event data

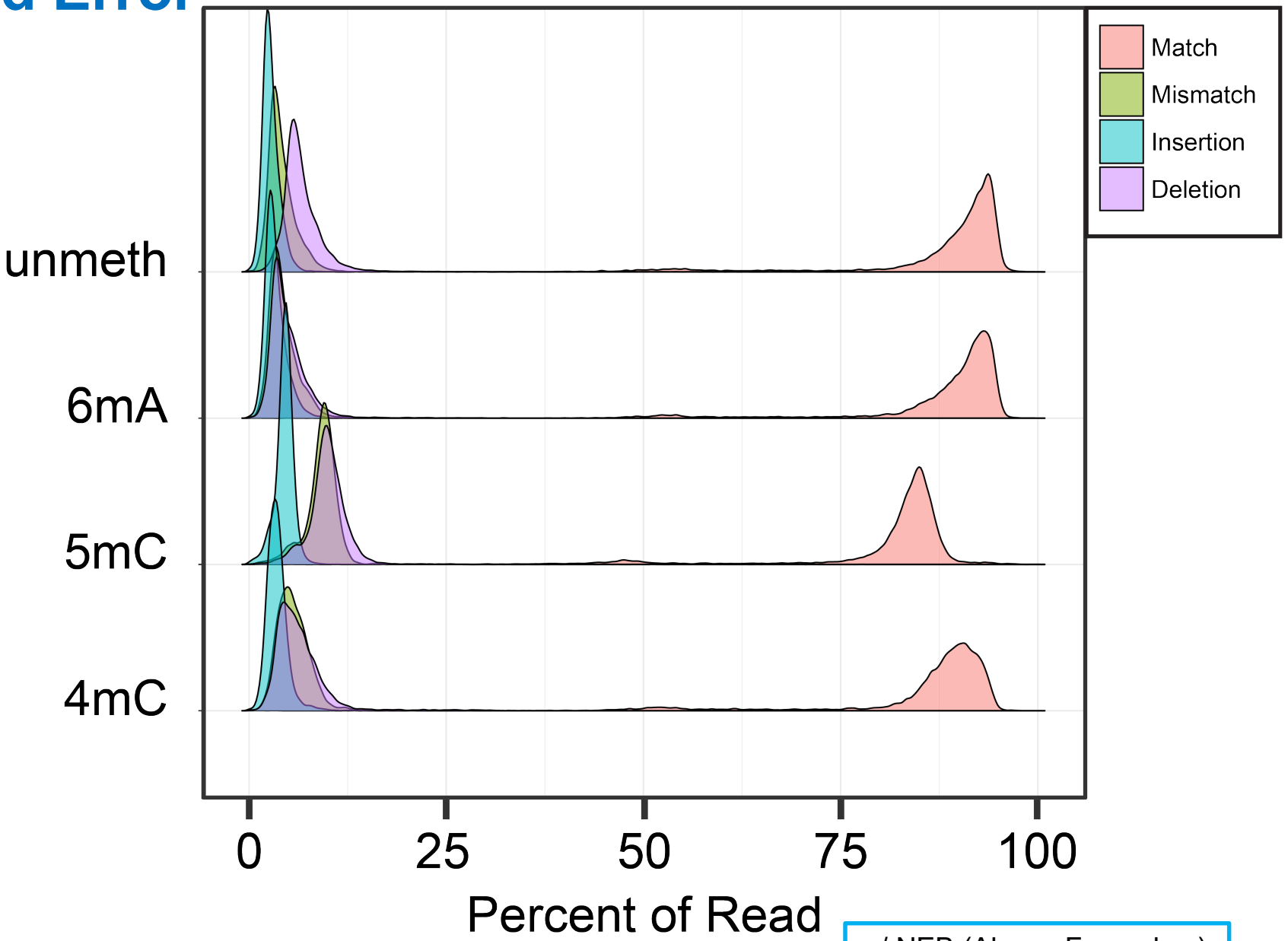


Assembly Using Signal to polish



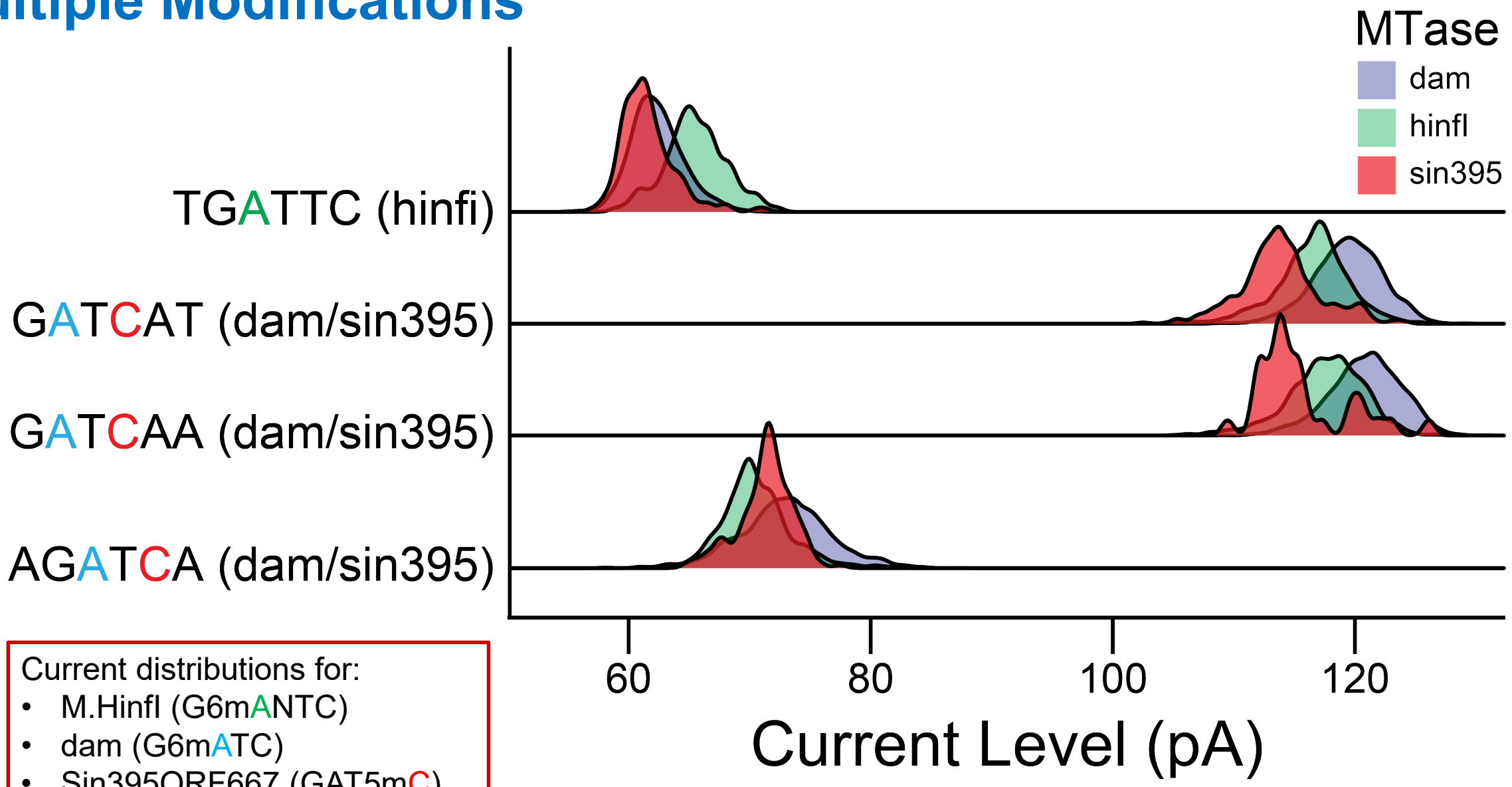
Nanopore: Methylated Error

- We sequenced samples from NEB ER2796 (E. Coli with KO of dam/dcm)
- Different methyltransferases are transformed in.
- Notably, mismatch error rate and deletions seem higher on methylated samples than unmethylated.
- The lower shift in 4mC and 6mA may be do to relative infrequency of those motifs.



w/ NEB (Alexey Fomenkov)

Multiple Modifications

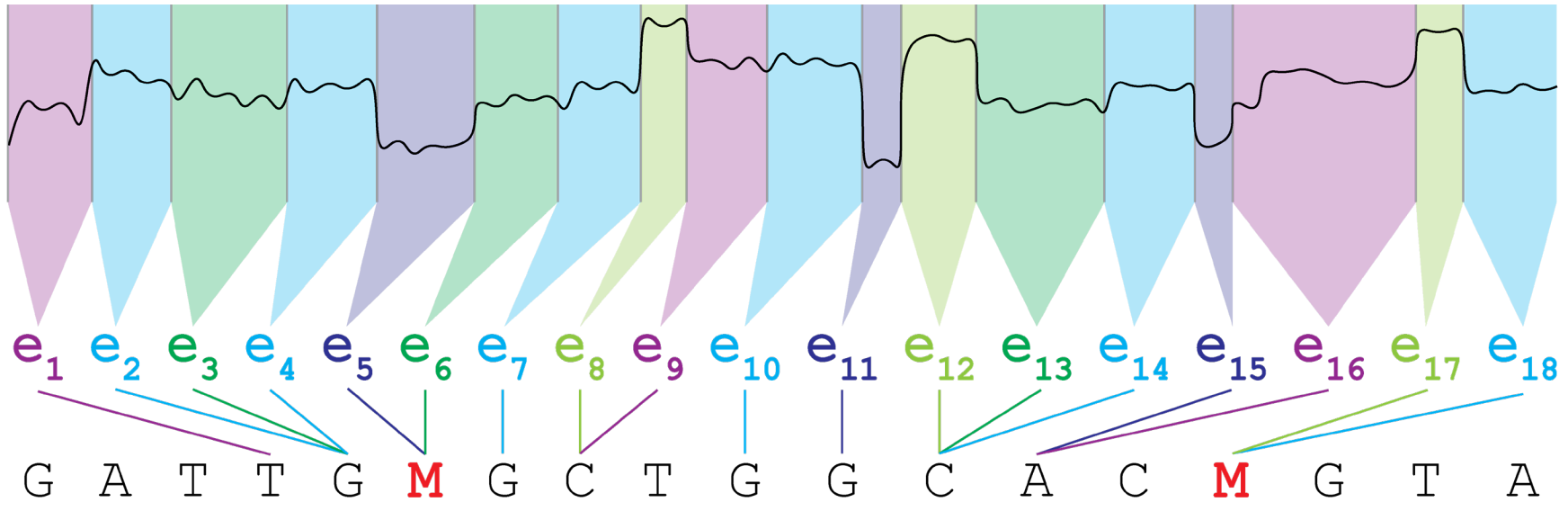


Current distributions for:
- M.HinfI (G6mANTC)
- dam (G6mATC)
- Sin395ORF667 (GAT5mC)

w/ Alexey Fomenkov (NEB)

Methylation Associated Error

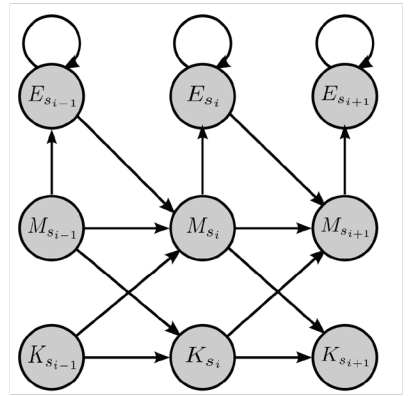
We can address this problem by training models specifically for methylation motifs, using a similar HMM scheme to align electrical data to a reference.



Calculate current models

TTGMG e_2 e_3 e_4
TGMGC e_5 e_6
GMGCT e_7
MGCTG e_8 e_9
ACMGT e_{17} e_{18}
CMGTA ...

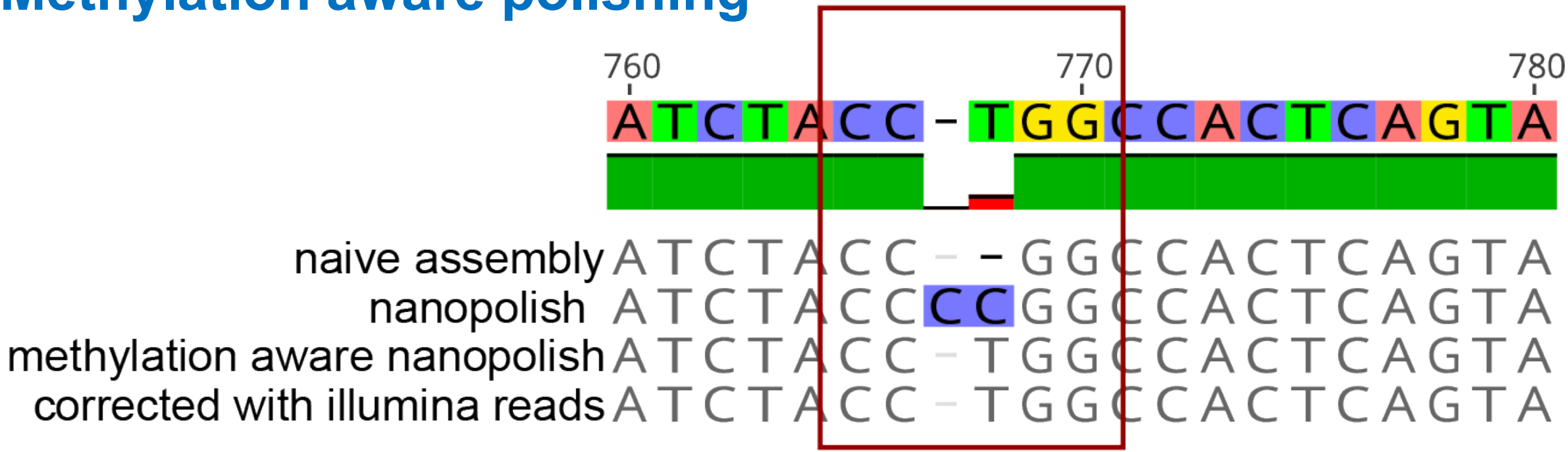
Train and apply new model parameters



Re-align

http://simpsonlab.github.io/2017/10/31/methylation-

Methylation aware polishing



models need to be trained on methylated k-mers in order to correct MTase motifs (dcm MTase= **CCWGG**)

Raw Assembly	Nanopolish Corrected	Methylation Aware Nanopolish Corrected
98.89%	99.57%	99.76%

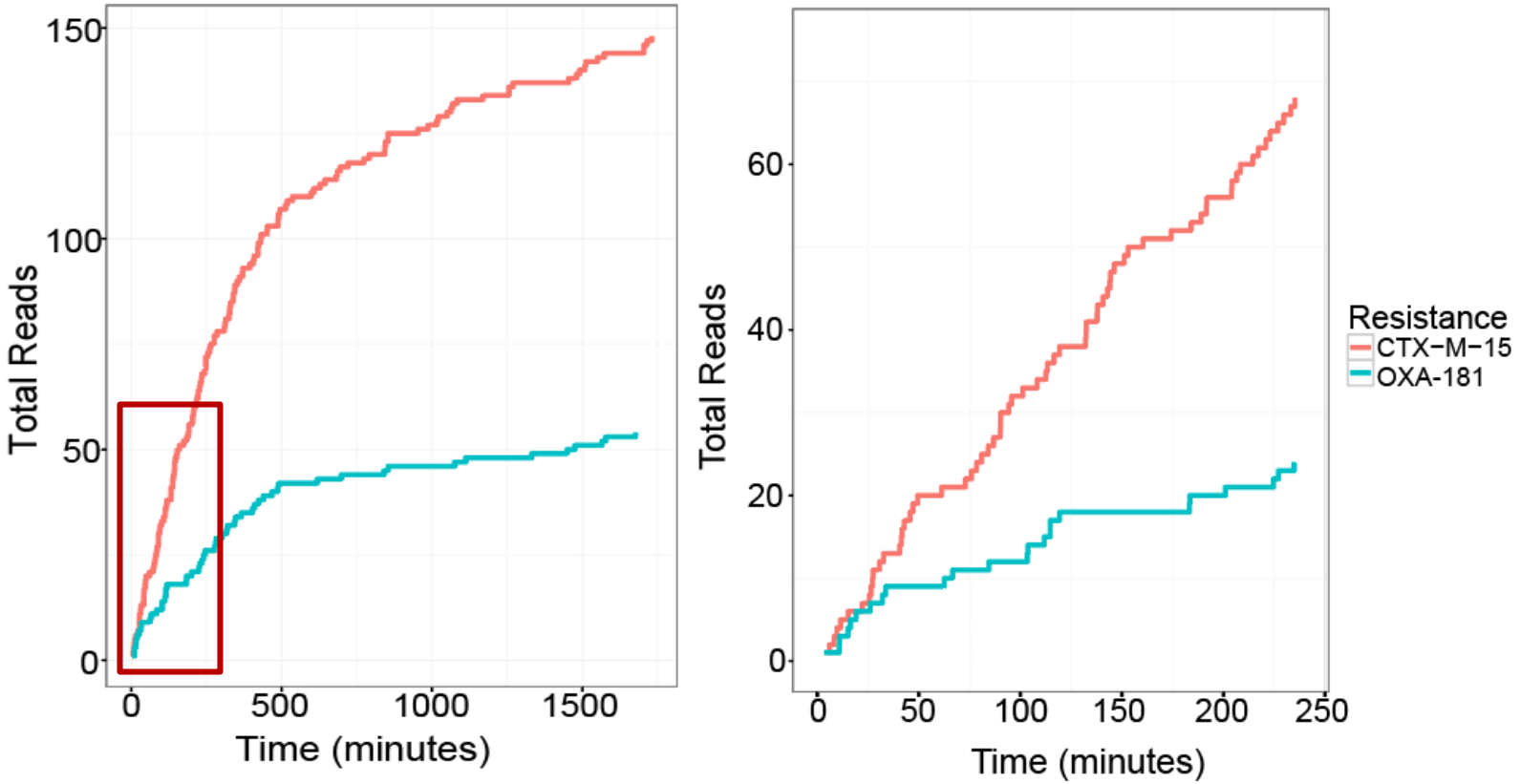
Differences report
Mutation or sequencing artifact?

>KLPN_133_tig00000001:3594157-3595237- coverage:100.00 score=2120 edit_distance=6

D	115	39(M)	A:	115	GAAAATGGTC	AAAAATGGT	homopolymer		premature stop at AA63
I	123	41(G)	:G	123	GTCGGgCGAGC	TCGGCGAGC	[unknown]		premature stop at AA89
S	294	98(L)	G:A	295	CGTCTaGCGTT	GTCTGGCGT	[unknown]		synonymous
S	688	230(W)	T:C	689	AAGCCcGGGCG	AGCCTGGGC	motif	CCTGG	AA230|W:R
D	946	316(W)	T:	946	GTACCGGTAC	TACCTGGTA	motif	CCTGG	premature stop at AA323
I	1049	350(Q)	:G	1049	CGACCgAGGCG	GACCAGGCG	motif	CCAGG	frame shift QAAV+:RGGR+

1. event type
2. WT base loc
3. Amino acid
4. base changes
5. ctg base loc
6. ctg context (5+m+5)
7. WT context (4+1+4)
8. context status
9. mutation impact

Building Diagnoses: Real-time Detection



Sample	OXA-181 detection time	CTX-M-15 detection time
4	4.8 min	2.8 min
8	10.3 min	3.6 min
9	10.73 min	9.37 min
10	0.99 min	1.00 min
12	13.07 min	6.04 min

Resistance identified within 15 minutes!

As reads can be identified as they come off the sequencer, we can identify AMR rapidly
Our retrospective analysis showed the resistance was identified for all of our isolates within 15 minutes

Summary

- Nanopore technology is full of potential for sequencing, but always choose the right tool for the right job. Often multiple approaches with complementary data yield the best results.
- Multiple bases affect the electrical signal from nanopores; rather than a problem, this can be an advantage, as each base is interrogated multiple times.
- Modifications to the primary DNA sequence (e.g. cytosine methylation) can be detected directly using nanopores
- Targeted sequencing with Cas9 allows for long reads in targeted regions, sidestepping issues of cost.
- Exogenous labeling allows simultaneous detection of chromatin and methylation state using nanopore sequencing

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