Long Read Sequencing

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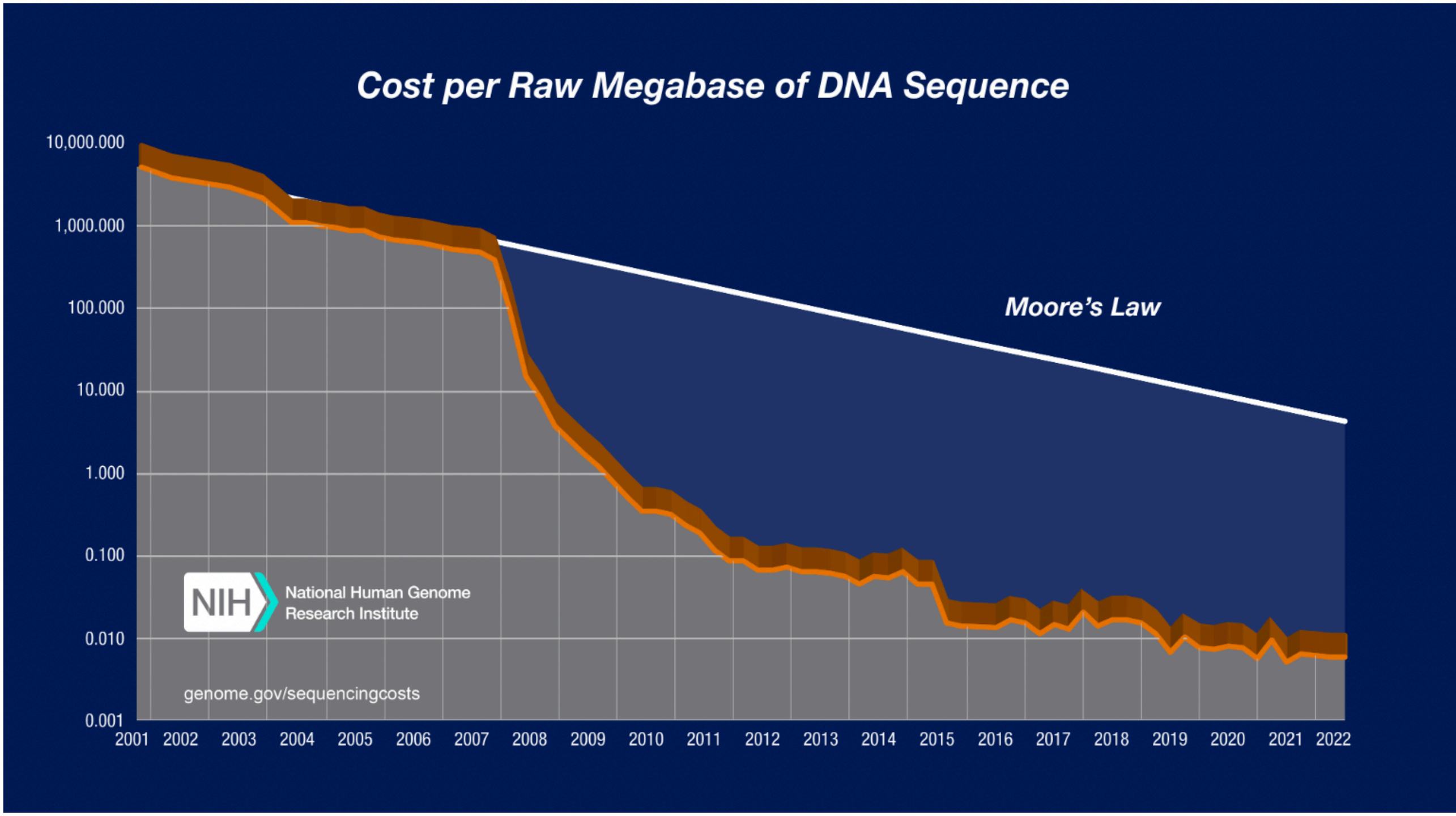
Advanced Sequencing Technologies and Applications course
Cold Spring Harbor Laboratory
2024

Significant advances in genome sequencing over last 20 years



Evolution of genome assemblies

- Initial references – very high quality – extremely expensive
- Period of lower quality Sanger assemblies (~2001-2007)
- Next gen assemblies (short read) – 2007- now
- Third generation – long read assemblies -2013/2014 –now – what can we do currently?
- T2T extremely complete genomes

Goodwin, McPherson and McCombie. Nat. Rev. Genetics. 2016

Short vs long reads

- Short read NGS has revolutionized resequencing
- *De novo* assembly is possible but not optimal with short reads
- Long reads improve the ability to do *de novo* assembly dramatically
- Even in organisms with a good reference, such as humans, resequencing misses many structural differences relative to the reference
- Plant genomes are very large in general
- There are significant structural differences between different strains of the same plant such as rice
- These structural differences contribute to salient biological differences

Advantages of Long Read length

Full scale of genetic variation

Repetitive regions

Structural variants

Enables higher quality alignments and assembly

Gapless genomes - T2T

??

The Telomere-to-Telomere Consortium

Long read sequencing of the hydatidaform mole CHM13 with multiple technologies

-PacBio HiFi

- ONT ultralong reads

- Illumina Arima Genomics Hi-C (Hi-C)

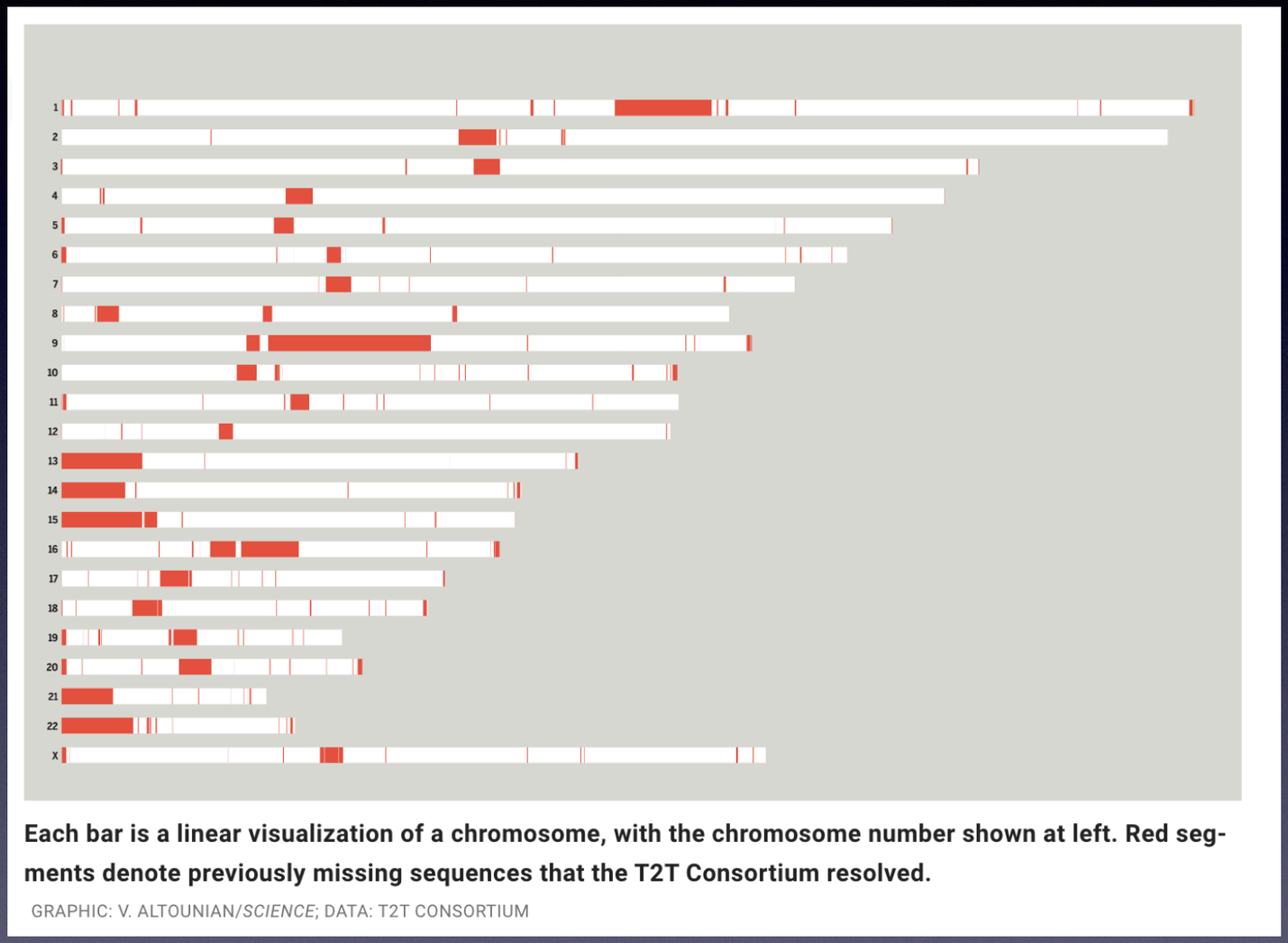
- BioNano optical maps

- single-cell DNA template strand sequencing (Strand-seq)

CHM13 reference

Vastly improves upon the previous "gold standard" reference genome GRCh38

- Introduces nearly 200 million base pairs of sequence
- 1956 new gene predictions, 99 of which are predicted to be protein coding
- Gapless assemblies for all chromosomes except Y
- Corrects errors in the prior reference
- Resolves highly repetitive/ complex regions



Each bar is a linear visualization of a chromosome, with the chromosome number shown at left. Red segments denote previously missing sequences that the T2T Consortium resolved.

GRAPHIC: V. ALTOUNIAN/*SCIENCE*; DATA: T2T CONSORTIUM

Filling the gaps

Laura M. Zahn

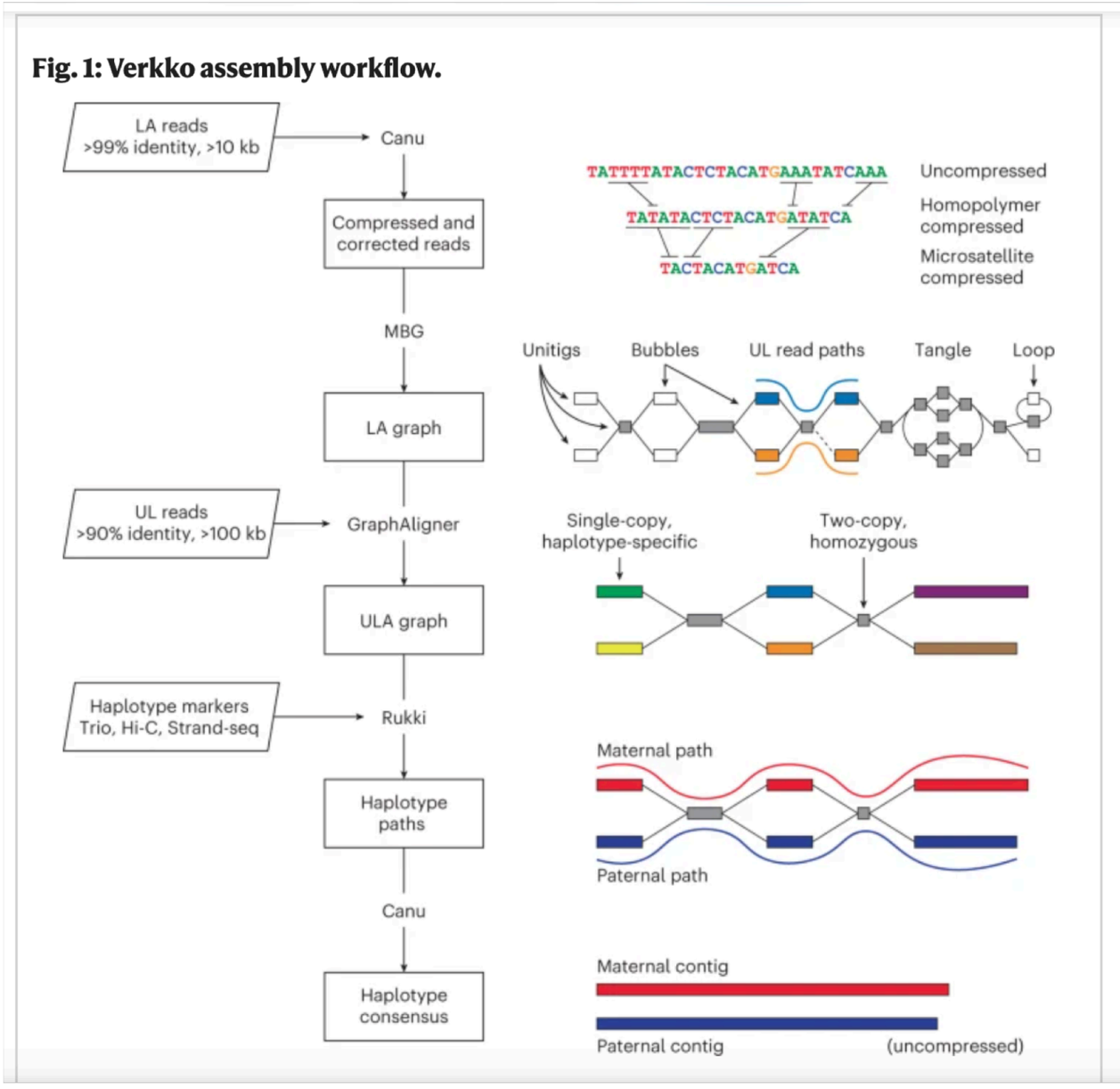
Science, 376 (6588), • DOI: 10.1126/science.abp8653

T2T phased diploid genomes

Improve variant detection compared to previous haploid references, simplify genotyping

Pan Genomes provide greater representation of diversity

Liao, WW., Asri, M., Ebler, J. *et al.* A draft human pangenome reference. *Nature* **617**, 312–324 (2023). https://doi.org/10.1038/s41586-023-05896-x



Rautiainen, M., Nurk, S., Walenz, B.P. *et al.* Telomere-to-telomere assembly of diploid chromosomes with Verkko. *Nat Biotechnol* (2023). https://doi.org/10.1038/s41587-023-01662-6

Limitations of long reads

- Cost
- Throughput*
- Accuracy*
- DNA amount required*
- DNA quality required
- Data storage footprint

*This is rapidly changing

Sample Input Requirements For Long Reads

Instrument	Initial DNA input	Latest DNA input
Pacbio Sequel HiFi	10ug	1ug
PacBio Revio	2ug	500ng (SPRQ Dec 2024)
Oxford Nanopore PromethION	5ug	1ug

For smaller genomes <1Gb less DNA (300-400ng) may be sufficient

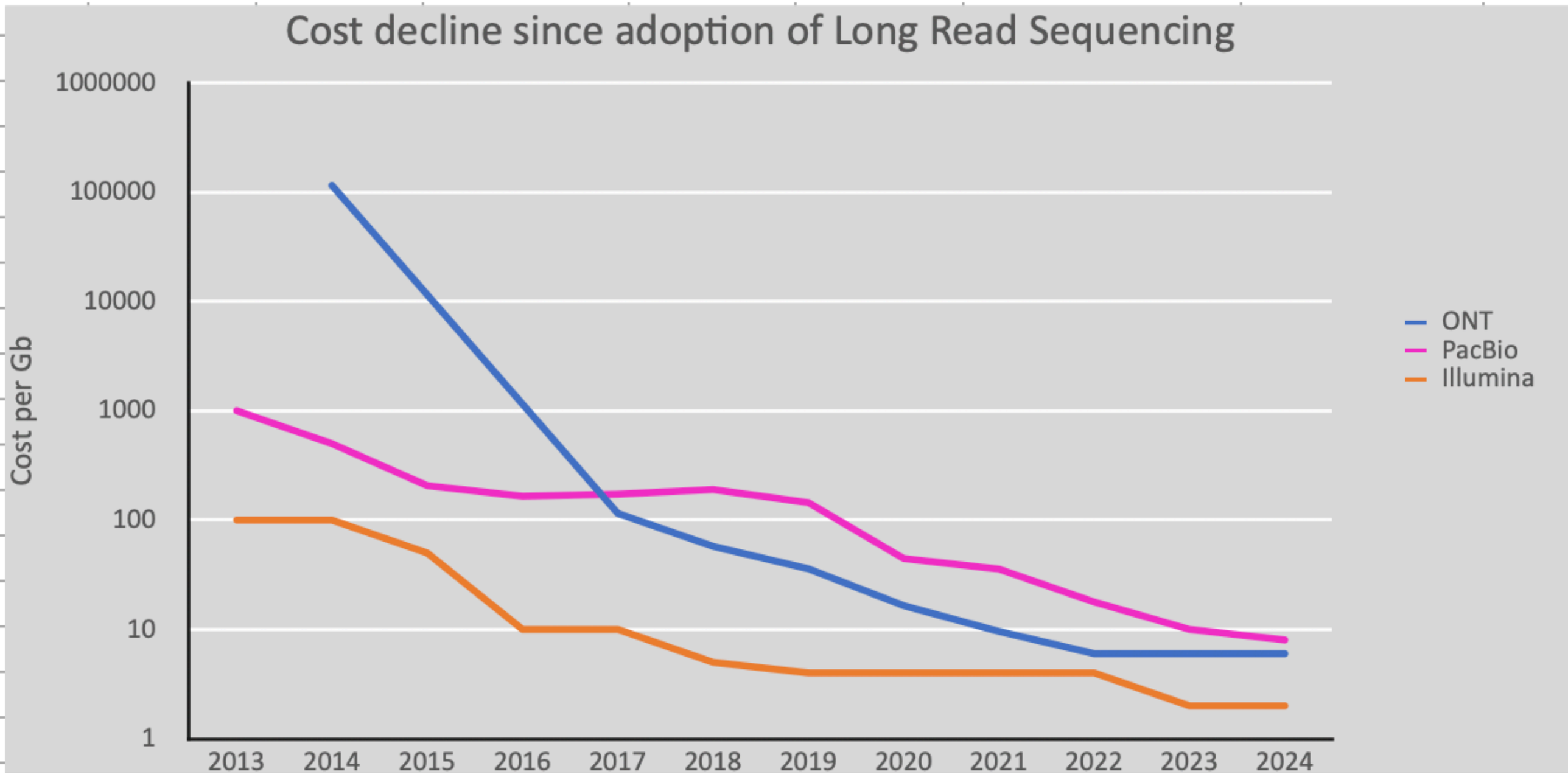
If PCR amplification is used, input may be as low as 5ng

Two “flavors” of long read sequencing

PACIFIC
BIOSCIENCES®

Oxford
NANOPORE
Technologies

Significant advances in long read sequencing over last 11 years



Platform Comparisons

Instrument	Avg Readlength	Yield (Gb)	Cost per Gb
Illumina NovaSeq X	300bp	8000	$2.00
PacBio Sequel II	15-20kb	30	$18.00
PacBio Revio	15-20kb	120	$8.00
Oxford Nanopore PromethION	40-80kb	150	$5.00

PACIFIC
BIOSCIENCES®

Pacific Biosciences Revio

Similar in size to Sequel

25M ZMW (1M Sequel I, 8M Sequel II)

Main focus is HiFi data

Runs 4 chips in parallel

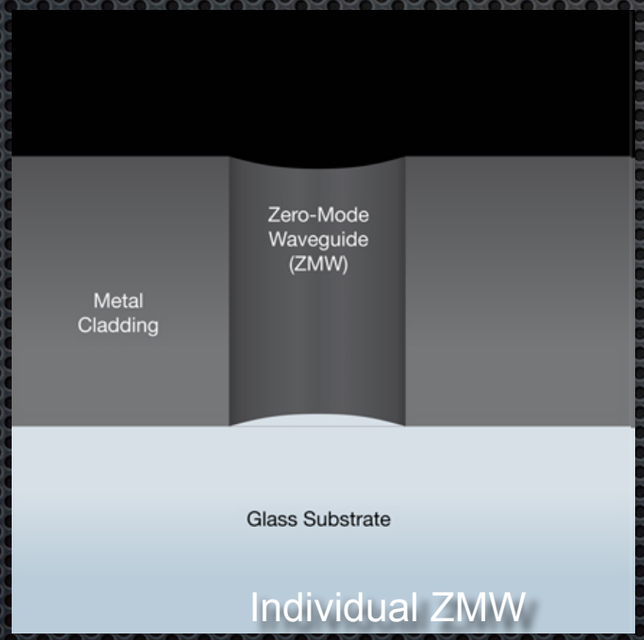
Estimated up to 3Tb of HiFi data per week

Zero-Mode Waveguides Are the Observation Windows

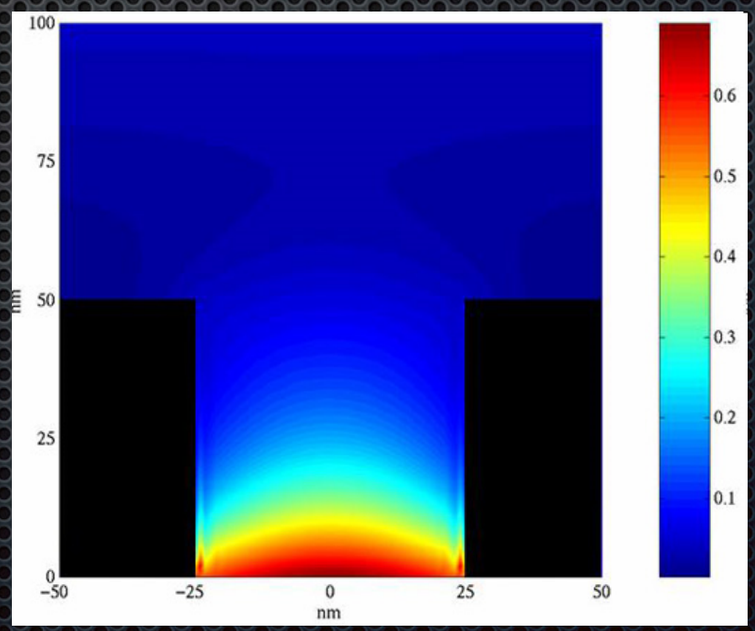
DNA sequencing is performed on SMRT™ Cells, each containing tens of thousands of zero-mode waveguides (ZMWs)

A ZMW is a cylindrical hole, hundreds of nanometers in diameter, perforating a thin metal film supported by a transparent substrate

The ZMW provides a window for observing DNA polymerase as it performs sequencing by synthesis



Individual ZMW

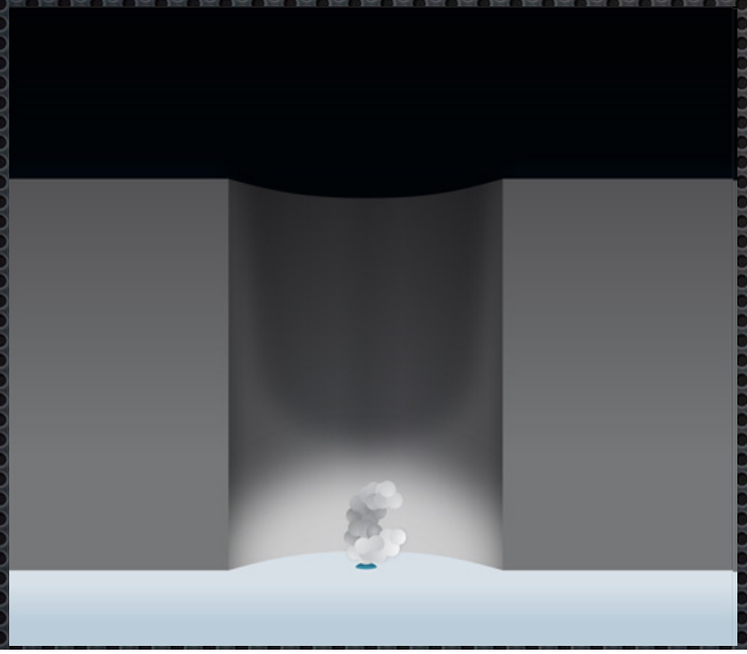


Laser light illuminates the ZMW

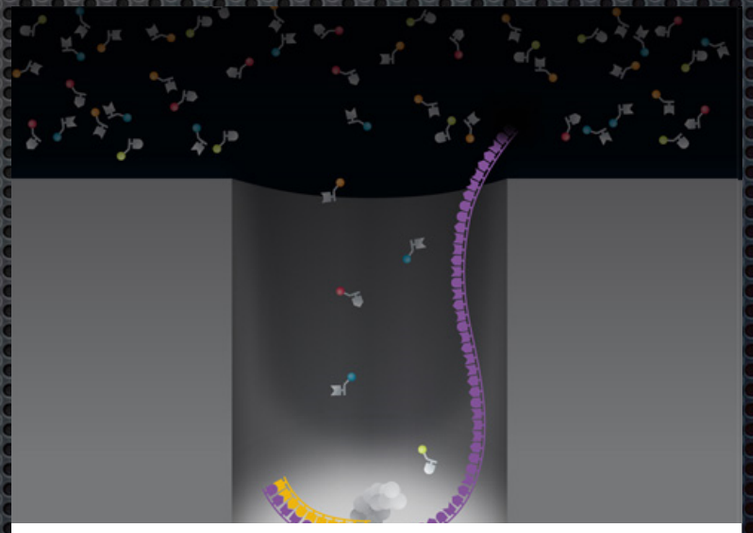
DNA Polymerase as a Sequencing Engine

A single DNA polymerase molecule is attached to the bottom of the ZMW

A single incorporation event can be identified against the background of fluorescently labeled nucleotides



ZMW with DNA polymerase

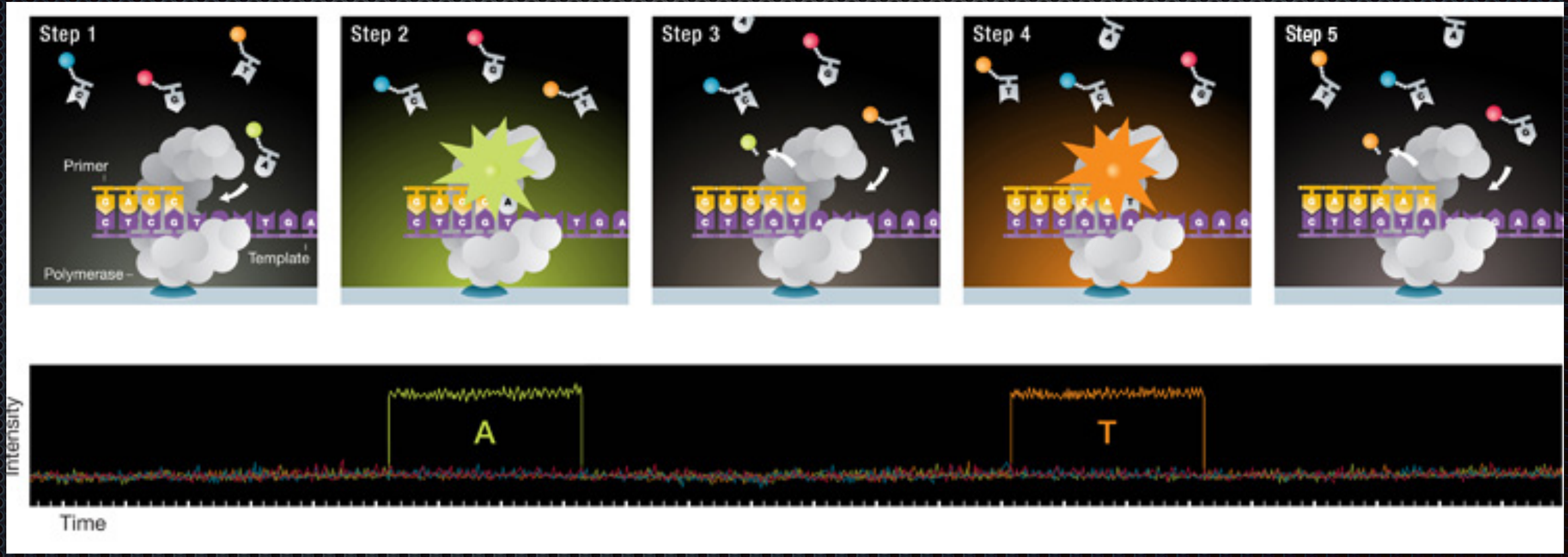


ZMW with DNA polymerase and phospholinked nucleotides

Processive Synthesis with Phospholinked Nucleotides

Enzymatic incorporation of the labeled nucleotide creates a flash of light, which is captured by the optics system and converted into a base call with associated quality metrics using optimized algorithms

To generate consensus sequence from the data, an assembly process aligns the different fragments based on common sequences



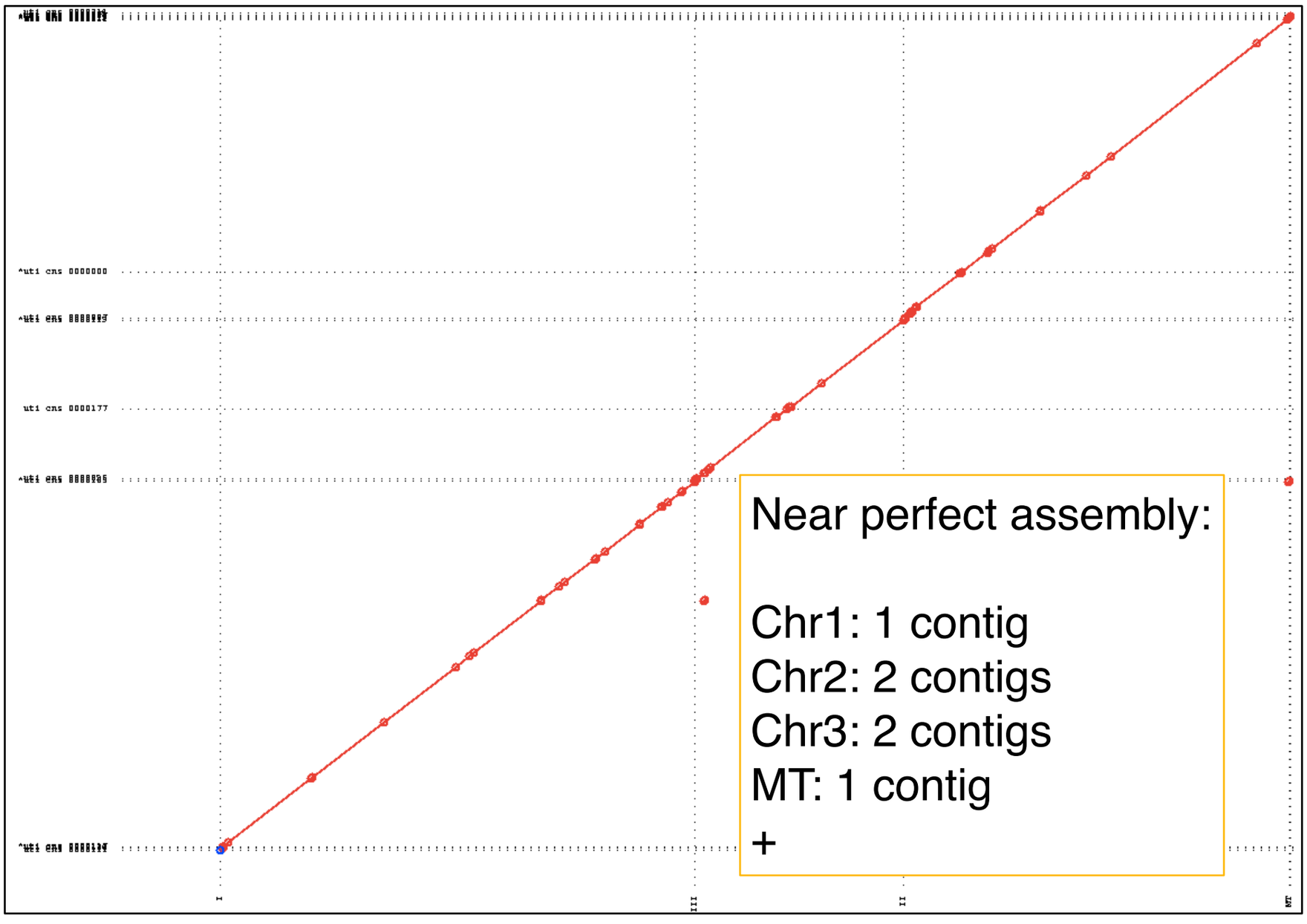
S. pombe dg21

ASM294 Reference sequence

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

PacBio assembly using HGAP + Celera Assembler

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



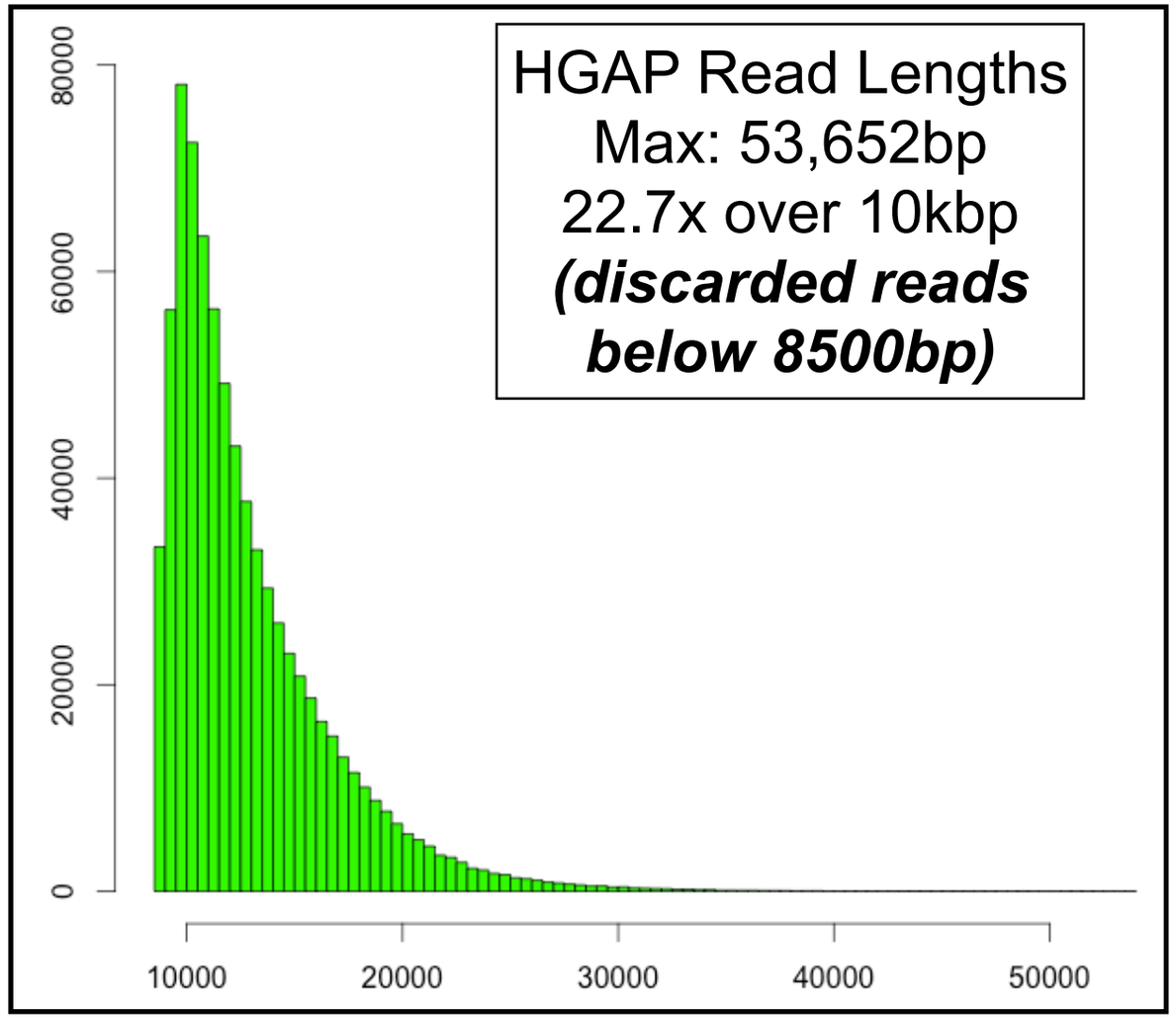
Near perfect assembly:

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

O. sativa pv Indica (IR64)

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

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

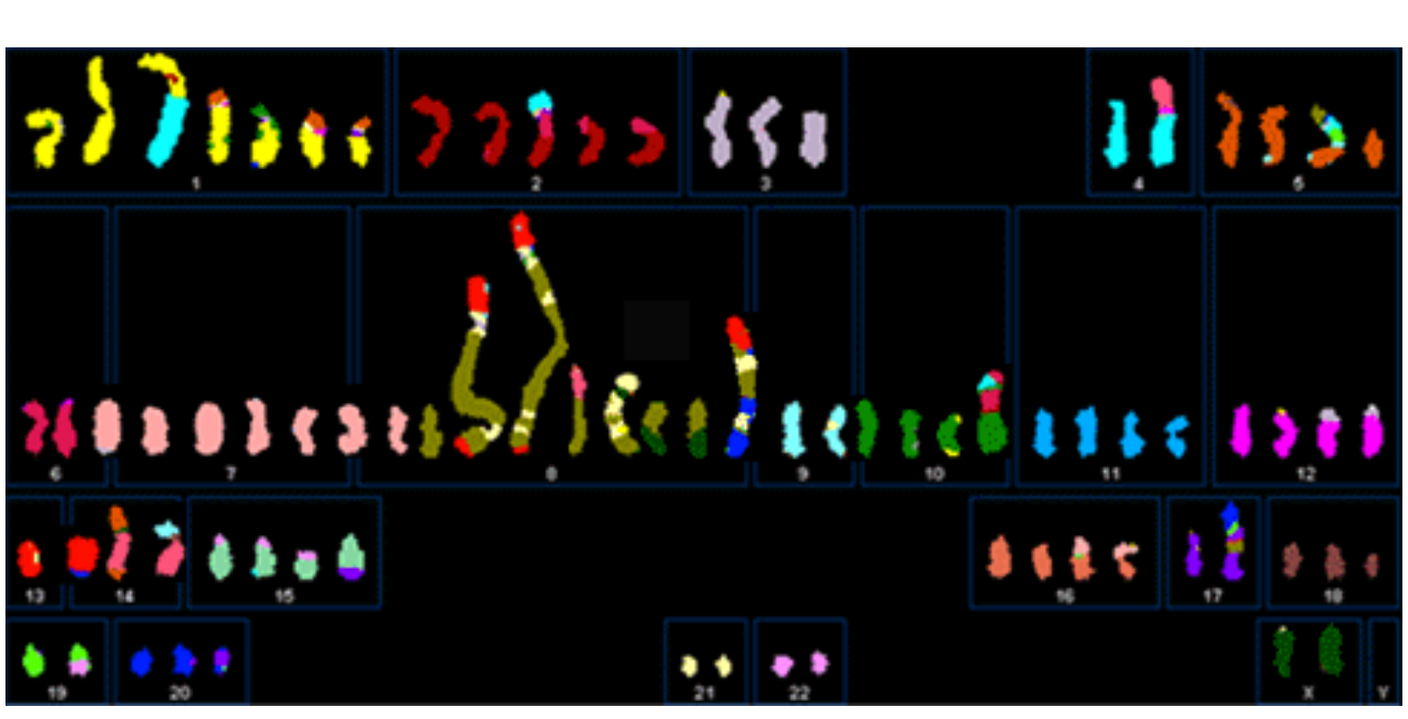


Structural Variations in SKBR3

SKRB3 cell line was derived by G. Trempe and L. J. Old in 1970 from pleural effusion cells of a patient, a white, Caucasian female

Most commonly used Her2-amplified breast cancer cell line

Often used for pre-clinical research on Her2-targeting therapeutics such as Herceptin (Trastuzumab) and resistance to these therapies.



(Davidson et al, 2000)

Nattestad, et al, Gen. Res. 2018

Assembly using PacBio yields far better contiguity

Number of sequences:
10,304

Total sequence length:
2.75 Gb

Mean: 266 kb

Max: 15 Mb

N50: 2.17 Mb

NG50: 1.86 Mb

Number of sequences:
748,955

Total sequence length:
2.07 Gb

Mean: 2.8 kb

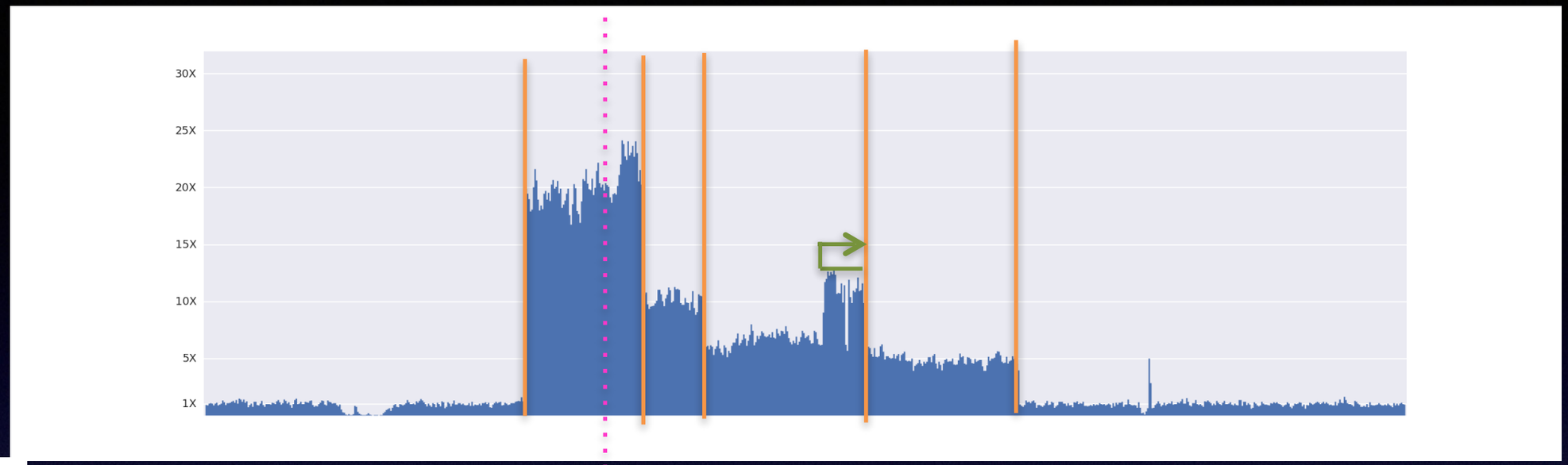
Max: 61 kb

N50: 3.3 kb

NG50: 1.9 kb

Her2

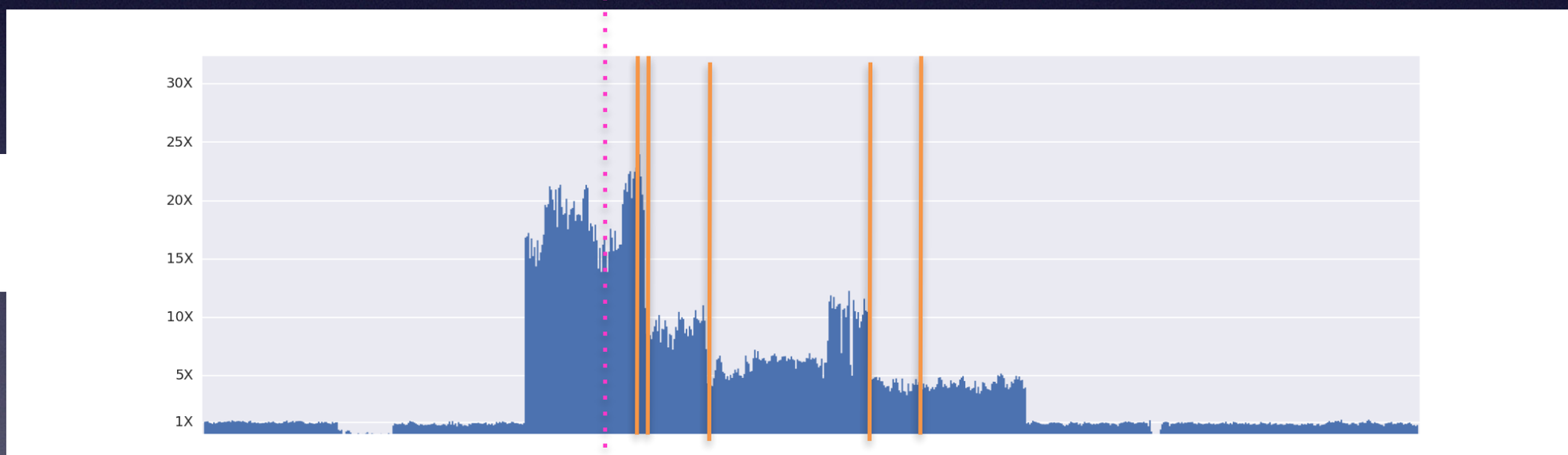
PacBio
73X @ 10kb



split reads

295 187 91 87 60

Illumina
120X @ 100bp



split reads

151,76 91 77 87

8 Mb

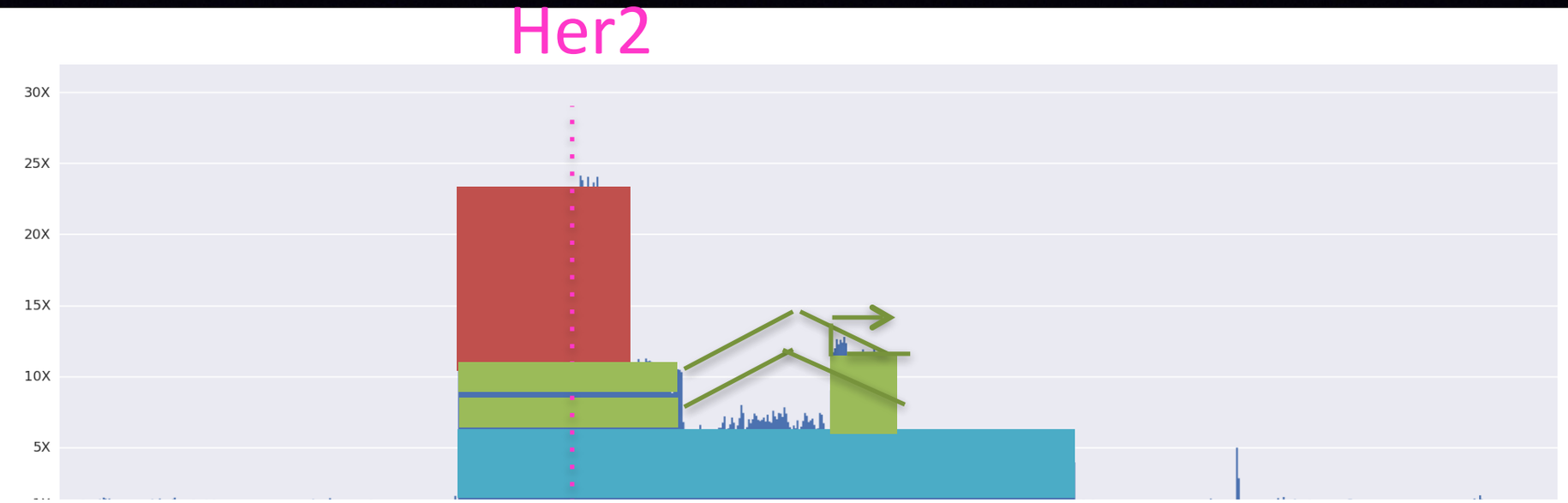
Green arrow indicates an inverted duplication.

False positive and missing Illumina calls due to mis-mapped reads (especially low complexity).

Cancer lesion reconstruction from genomic threads

PacBio

chr17



By comparing the proportion of reads that are spanning or split at breakpoints we can begin to infer the history of the genetic lesions.

1. Healthy diploid genome
2. Original translocation into chromosome 8
3. Duplication, inversion, and inverted duplication within chromosome 8
4. Final duplication from within chromosome 8

PacBio errors are randomly distributed

ATGCTCTTCGATCGATGCTGCTAGCTAGCTAGCTACTAGCTATCCGATCCTACTGACTTACTATGCT

ATGCTGTTCGATCGATGCTGCTAGCTAGCTAGCTACTAGCTATCCGATCCTACTGACTTACTATGCT

ATGCTCTTCGATCGATGCTGCTCGCTAGCTAGCTACTAGCTATCCGATCCTACTGACTTACTATGCT

ATGCTCTTCGATCGATGCTGCTAGCTAGCTAGCTACTAGCTATCAGATCCTACTGACTTACTATGCT

ATGCTCTTCGATCGATGCTGCTAGCTAGCTAGCTACTAGCTATCGGATCCTACTGACTTACTATGCT

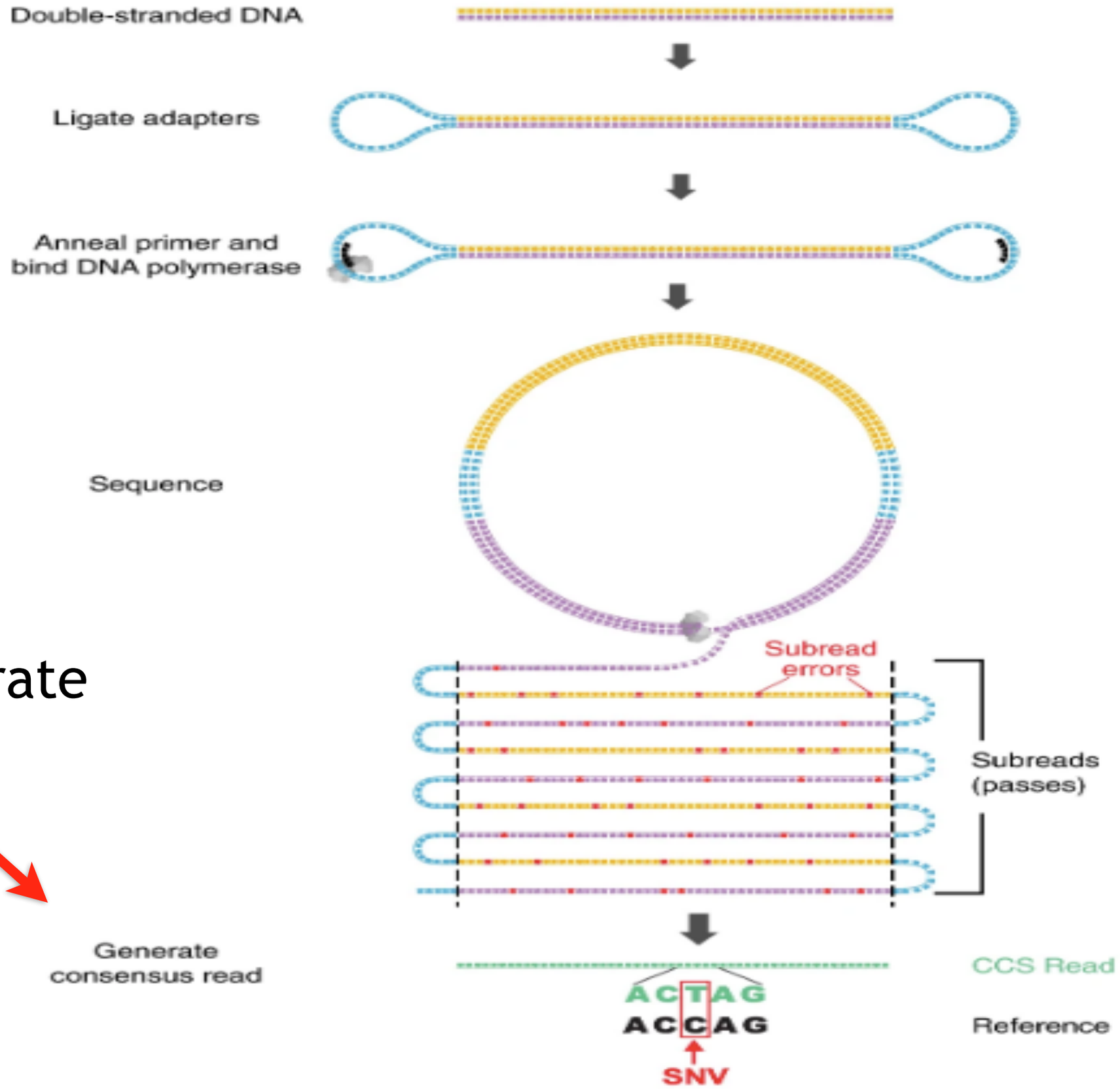
ATGCTCTTCGATCGATGCTGCTAGCTAGCTAGCTACTAGCTATCCGATCCTACTGACTTACTATGGT

ATGCTCTTCGATCGATGCTGCTAGCTAGCTAGCTACTAGCTATCCGATCCTACTGACTTACTATGCT

Enough coverage makes error drop out

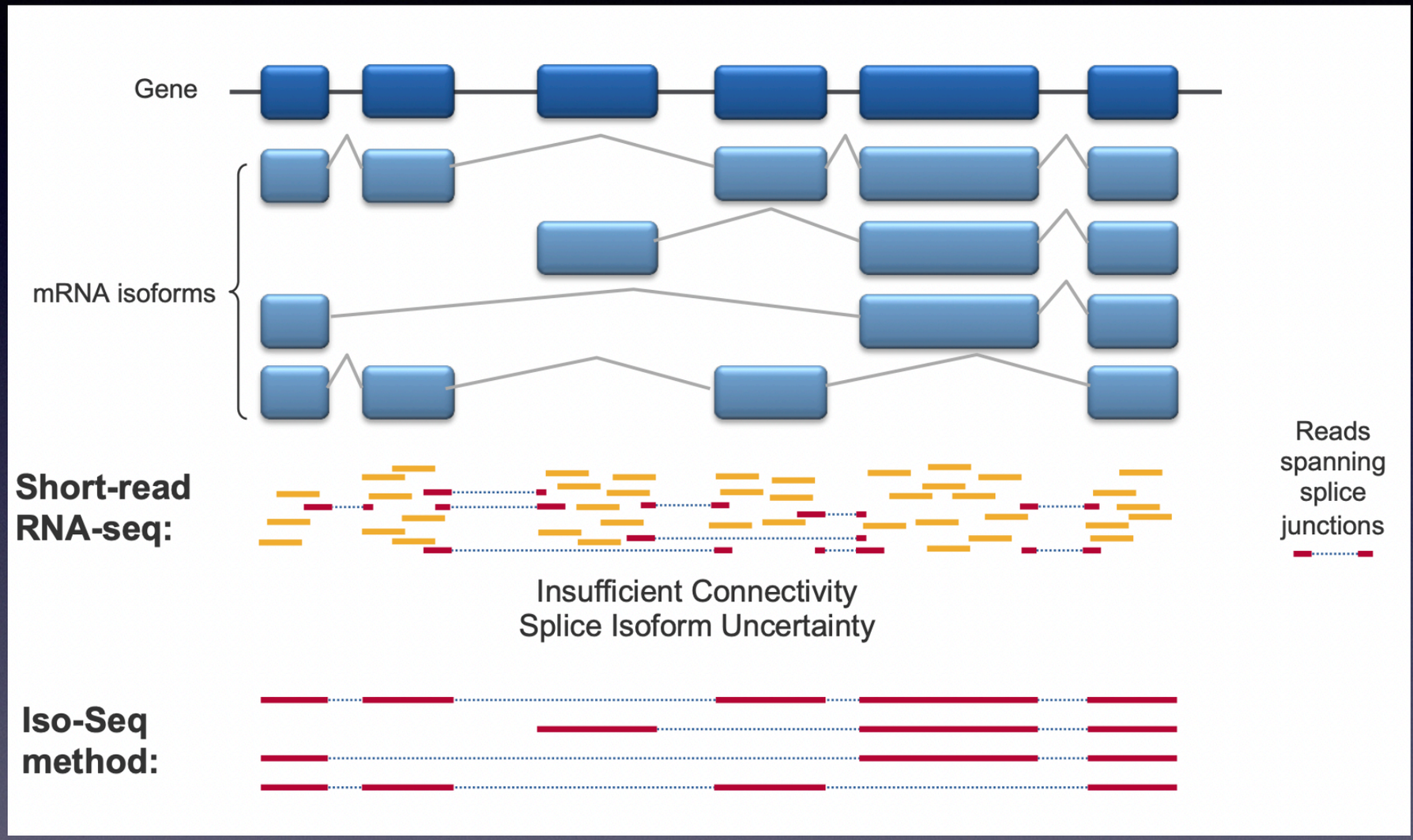
PacBio CCS “HiFi” for longer (~15-20kb) fragments

99.99% Accurate



From Wenger et al (2019) Nature Biotechnology

Benefits of long read transcripts



Long read transcripts provide complete isoform information, enables identification of alternative splicing, fusion events, and allows for isoform level phasing

PacBio IsoSeq transcript sequencing

Value	Analysis Metric
58,562,284	Reads
57,139,473	Reads with 5' and 3' Primers
57,100,348	Non-Concatamer Reads with 5' and 3' Primers
57,054,271	Non-Concatamer Reads with 5' and 3' Primers and Poly-A Tail (FLNC Reads)
1,472	Mean Length of FLNC Reads
1	Unique Primers
57,139,473	Mean Reads per Primer
57,139,473	Max. Reads per Primer
57,139,473	Min. Reads per Primer
1,422,811	Reads without Primers
97.43%	Percent Bases in Reads with Primers
97.57%	Percent Reads with Primers

Kinnex kits- high accuracy, concatenation increases throughput

Transcript Mapping and Classification

Value	Analysis Metric
1,197,157	Number of mapped unique isoforms
49,648	Number of mapped unique loci

Oxford NANOPORE Technologies

PromethION

24 independent flowcells

500bp/s sequencing speed

3000 pores per flowcells = 144,000 pores (fully loaded) (MinION cells 512 pores)

On board single or duplex basecalling

>140Gb in CSHL hands

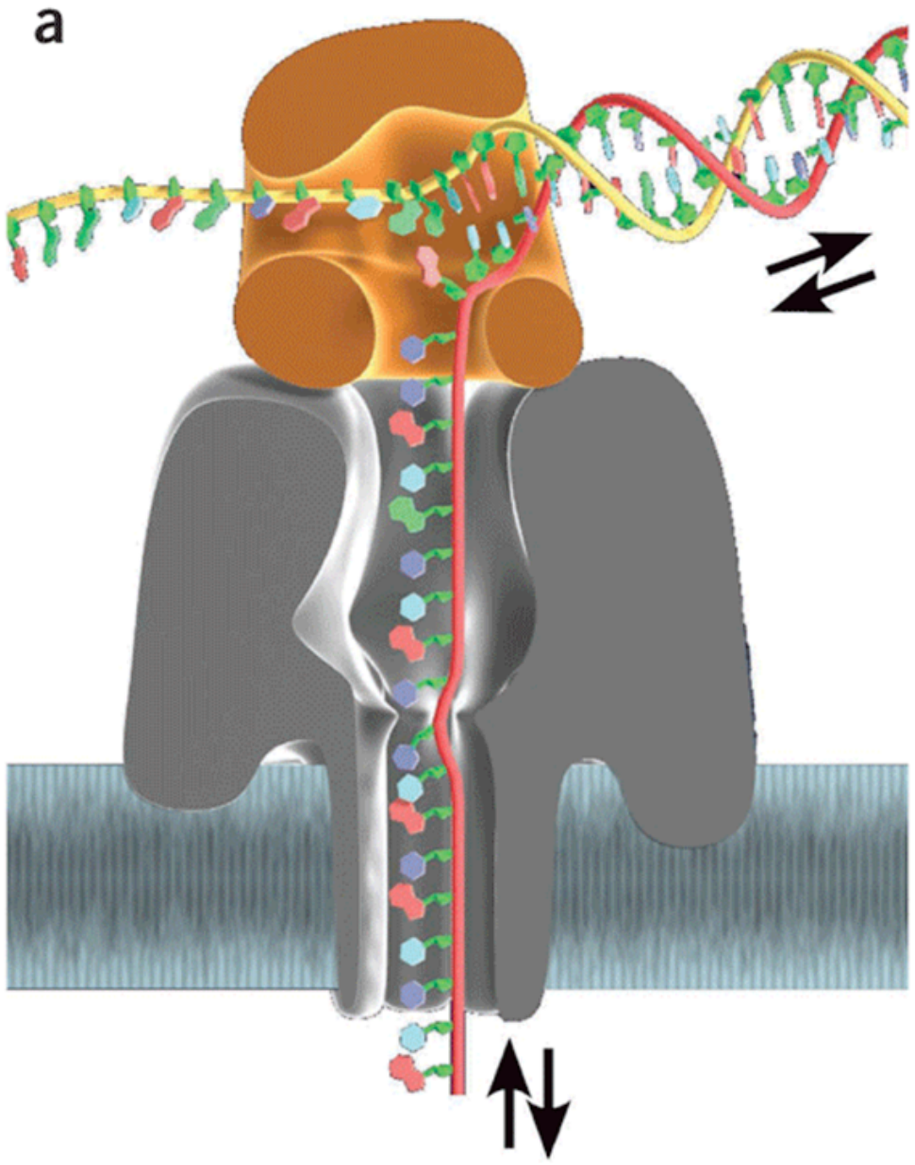
>100M cDNA reads

Up to ~5 Tb fully loaded in one week

Sequencing "flavors" include:

Ligation based - standard methods for gDNA
Q20 - enables higher accuracy including duplex
Barcoding - allows multiplexing up to 96 sample
16S - enables 16S metagenome sequencing
PCR sequencing - long-range PCR for low mass samples
Cas9 - enables Cas9 mediated target enrichment
Rapid - enables library prep <2hrs w/o mechanical shearing
Ultra-long - enables N50s up to 100kb
Native barcoding - PCR-free barcoding to preserve epigenetic marks
Field kit - enables sequencing in the field w/o cold chain
Short fragment - enables sequencing of fragments <1000bp

Oxford Nanopore relies on CsgG and a non-destructive motor protein



Cis side voltage drives DNA through pore

Motor protein mediates DNA unwinding and translocation speed

Ions flow through the pore to change membrane potential

Small changes in measured voltage are translated into k-mers

Nature Biotechnology 30, 326–328 (2012) doi:10.1038/nbt.2181Published online 10 April 2012

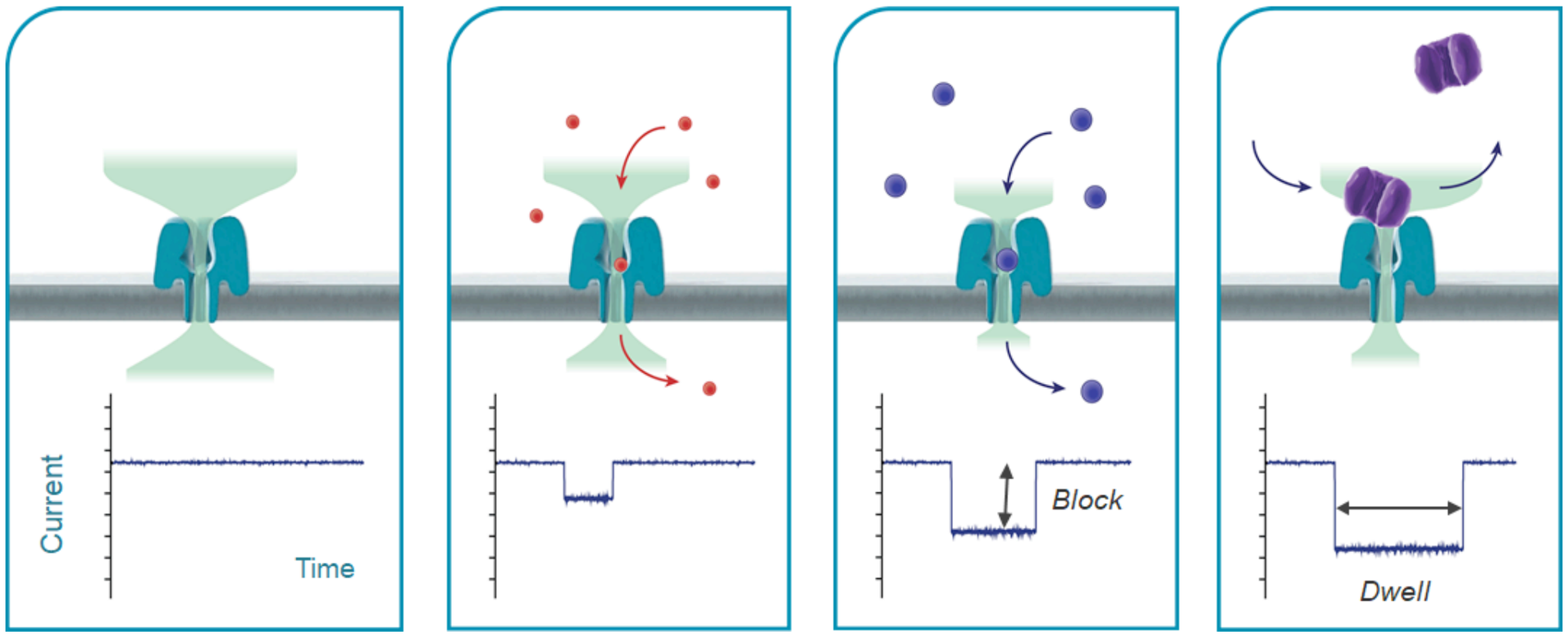
Nanopore Sensing Summary

Nanopore = ‘very small hole’

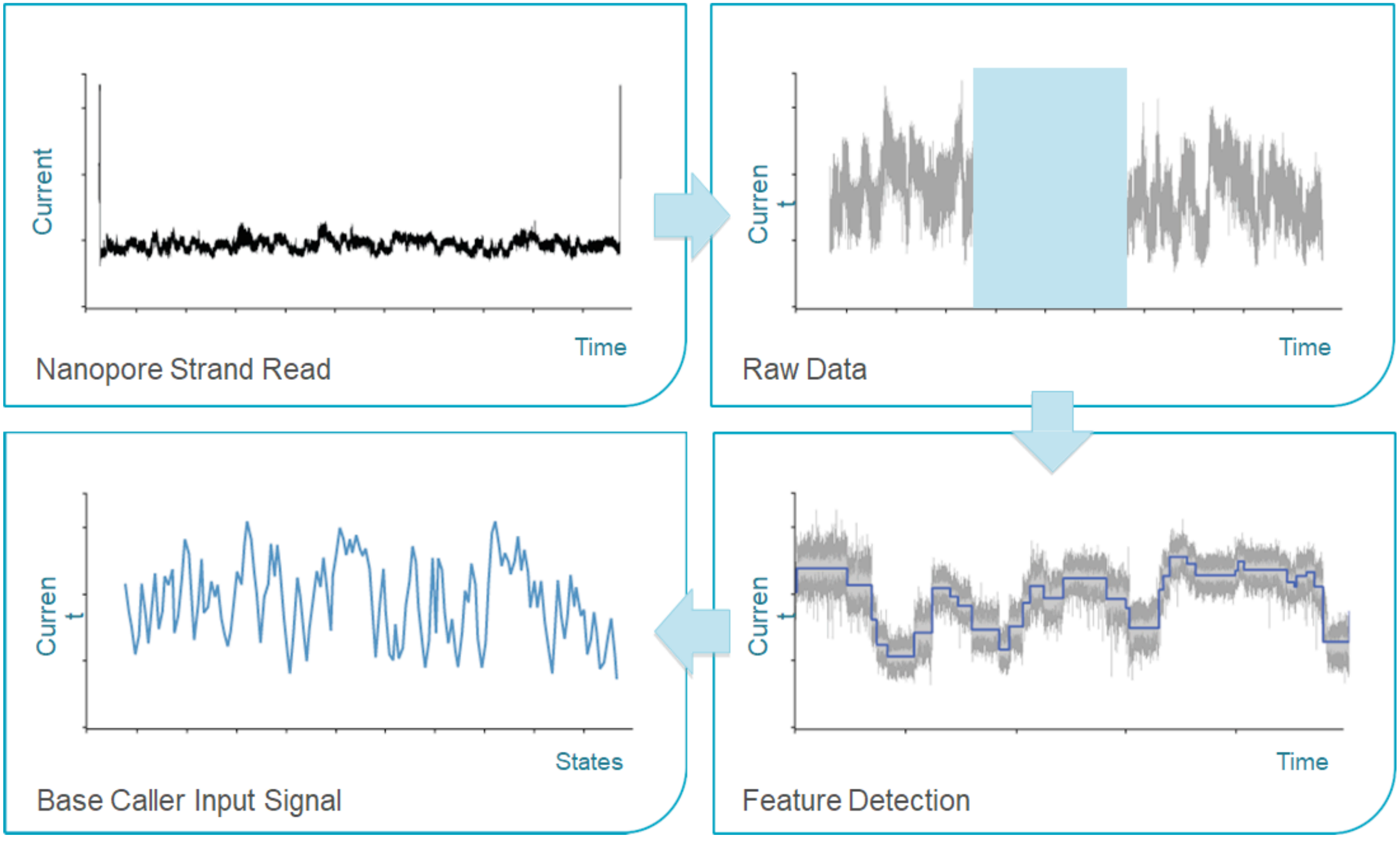
Ionic current flows through the pore Introduce analyte of interest into the pore

Identify target analyte by the characteristic disruption or block to the electrical current

Block or ‘State’, Dwell, Noise



Raw Data and Data Reduction



Nanopore errors are (mostly) randomly distributed

ATGCTCTTCGATCGATGCTGCTAGCTAGCTAGCTTTTTTTTTTCCGATCCTACTGACTTACTATGCT

ATGCTGTTCGATCGATGCTGCTAGCTAGCTAGCTTTTTT CCGATCCTACTGACTTACTATGCT

ATGCTCTTCGATCGATGCTGCTCGCTAGCTAGCTTTTTTTTT CCGATCCTACTGACTTACTATGCT

ATGCTCTTCGATCGATGCTGCTAGCTAGCTAGCTTTTTTTTTCAGATCCTACTGACTTACTATGCT

ATGCTCTTCGATCGATGCTGCTAGCTAGCTAGCTTTTTTTT CGGATCCTACTGACTTACTATGCT

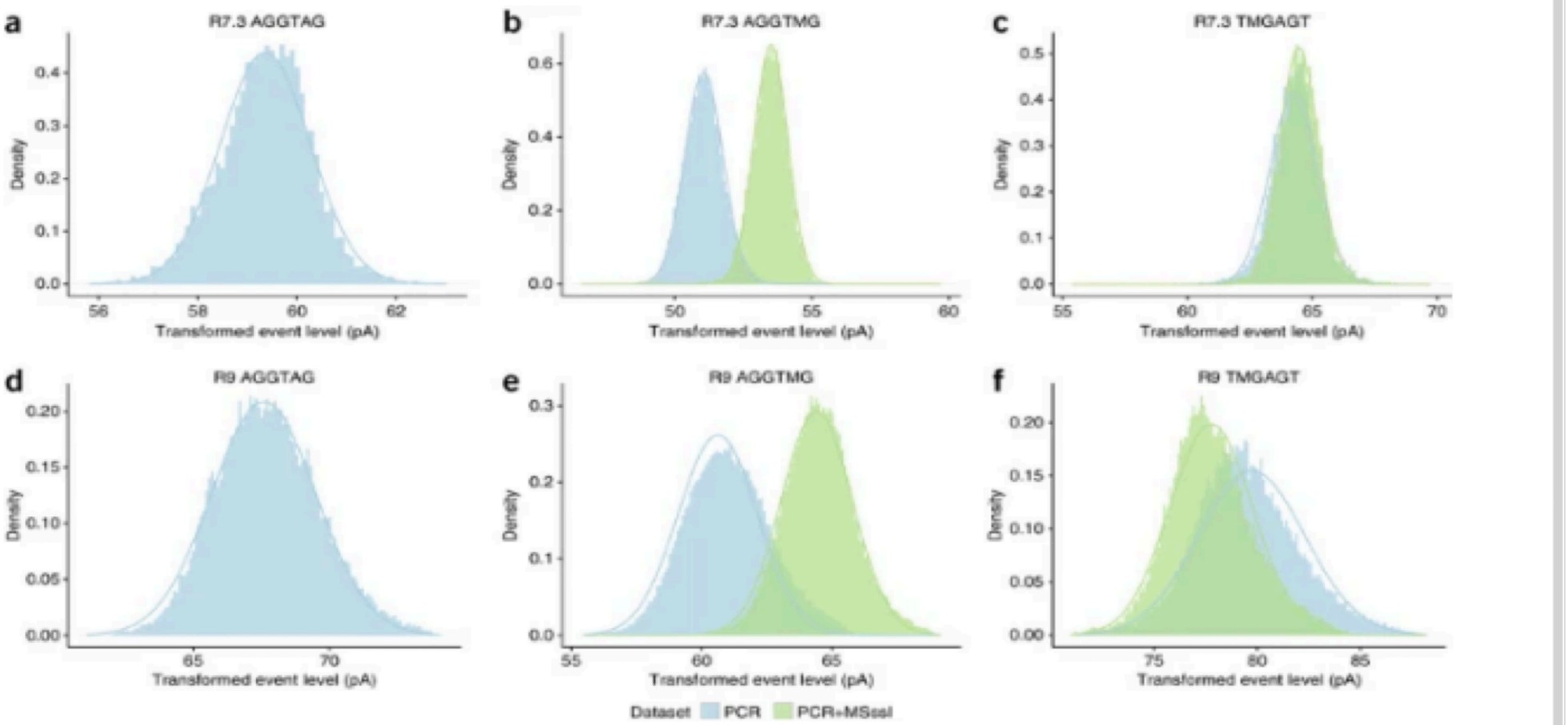
ATGCTCTTCGATCGATGCTGCTAGCTAGCTAGCTTTTTTTT CCGATCCTACTGACTTACTATGGT

ATGCTCTTCGATCGATGCTGCTAGCTAGCTAGCTTTTTTTT CCGATCCTACTGACTTACTATGCT

Enough coverage makes error (mostly) drop out

Simpson JT, Workman RE, Zuzarte PC, David M, Dursi LJ, Timp W. “Detecting DNA cytosine methylation using nanopore sequencing.” Nat Methods. 2017;14(4):407-410. doi:10.1038/nmeth.4184

Figure 1: Differences in event distribution between methylated and unmethylated 6-mers in nanopore sequencing data.

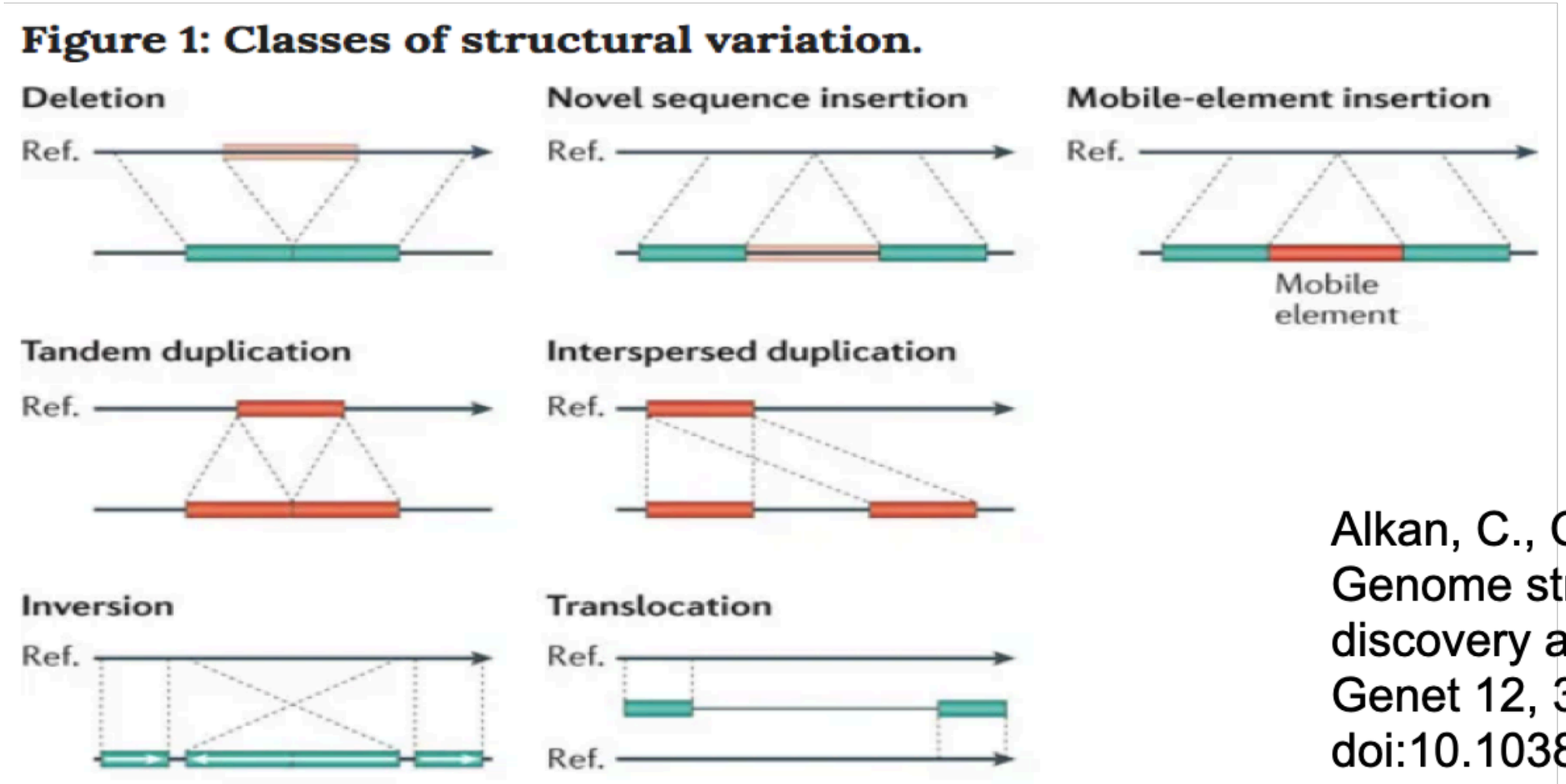


(**a**–**c**) Event distribution for the three sample 6-mers AGGTAG (**a**), AGGTMG (**b**) and TMGAGT (**c**) using R7.3 pore data from PCR-amplified *E. coli* DNA. 'M' represents 5-mC. (**d**–**f**) Data from the R9 pore for the AGGTAG (**d**), AGGTMG (**e**) and TMGAGT (**f**) 6-mers from PCR-amplified *E. coli* DNA. Data from M.SssI-methylated DNA are in green, and data from untreated DNA are in blue. Solid lines indicate Gaussian fits. Events are from the template strand and have been transformed to account for per-read differences with respect to the model (Supplementary Note).

Nanopores are sensitive to DNA modifications which alter the electrical current as the nucleotide transduces the pore. Simpson et al trained a HMM to distinguish signal differences between 5methylC and unmethylated cytosines. They used synthetically modified 6 mers from bacteria to test the shift of methylation at different positions.

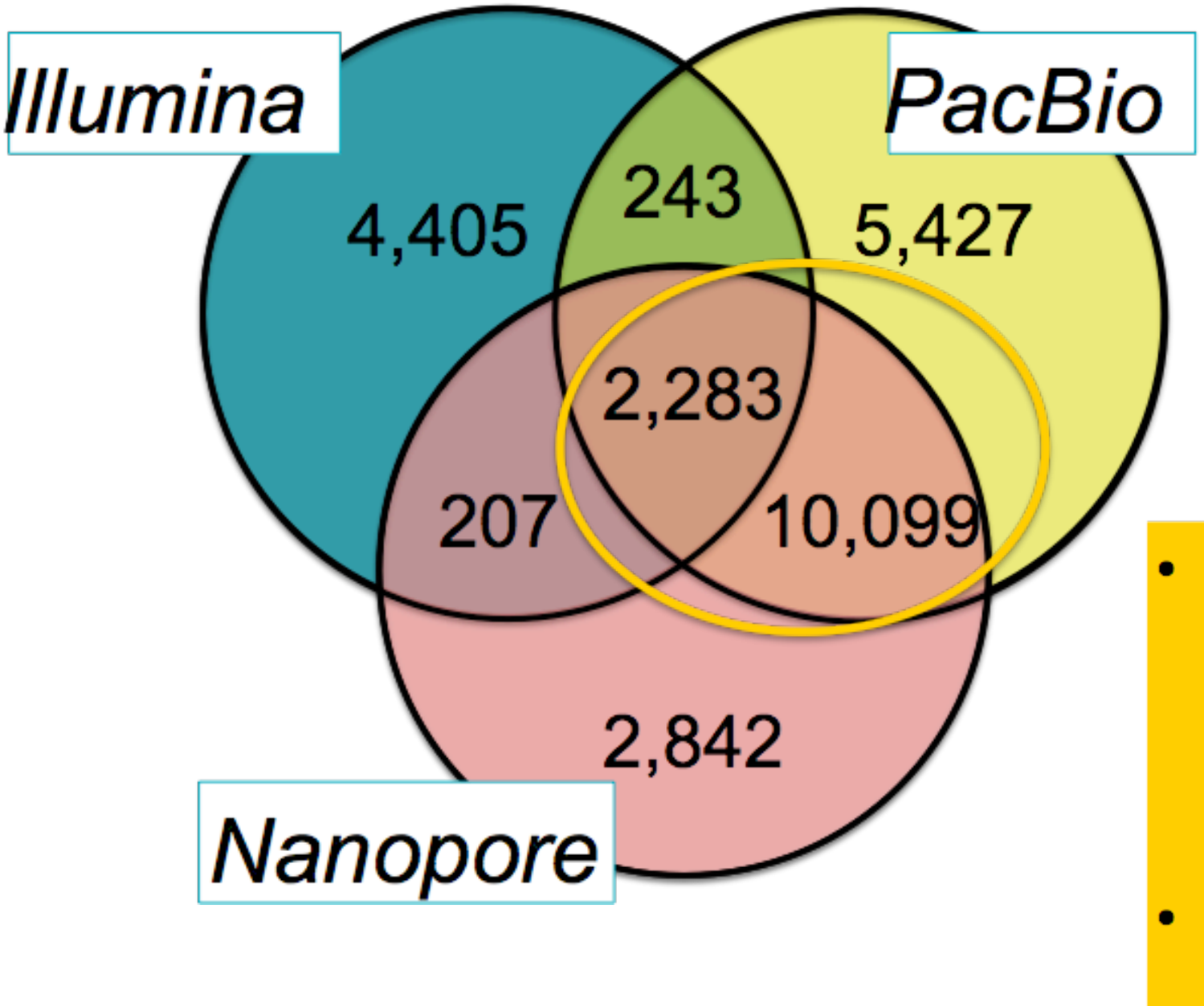
Significance of Structural Variants

- Often missed by short read approaches
- May uncover causative mutations underlying disease, especially where typical screening has failed
- SVs account for much more variation in the genome than SNPs
- SVs can alter methylation and gene expression
- However, the functional impact of most SVs remains unknown



Alkan, C., Coe, B. & Eichler, E. Genome structural variation discovery and genotyping. Nat Rev Genet 12, 363–376 (2011) doi:10.1038/nrg2958

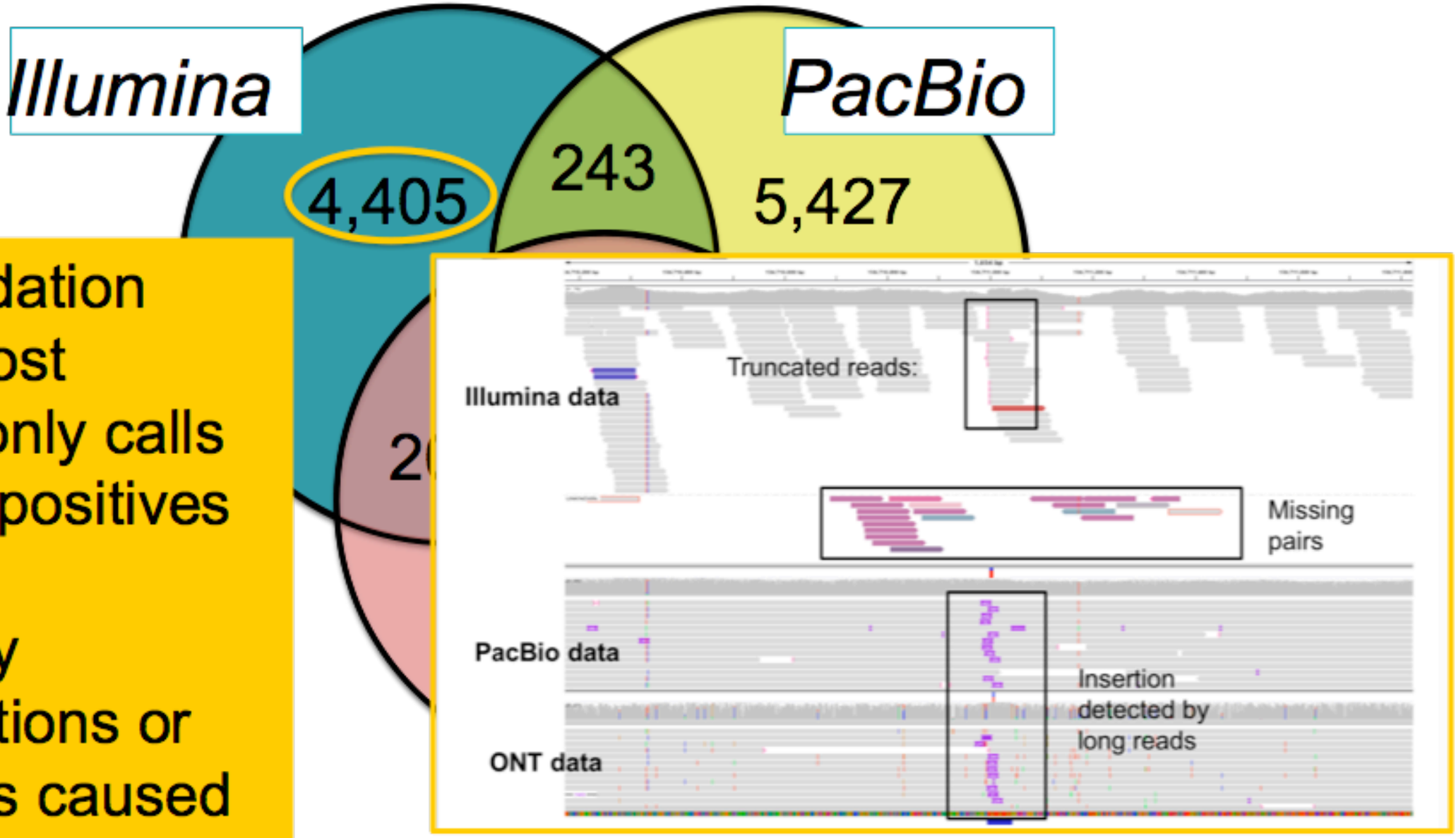
Structural Variant Comparison of SKBR3



- Strong concordance between long read platforms
- Substantially more variants than detected by short reads

(Hicks et al, 2006,

Structural Variant Comparison of SKBR3



- PCR validation shows most Illumina-only calls are false positives

- Especially translocations or inversions caused by smaller insertions or deletions

Preliminary Structural Variations Analysis

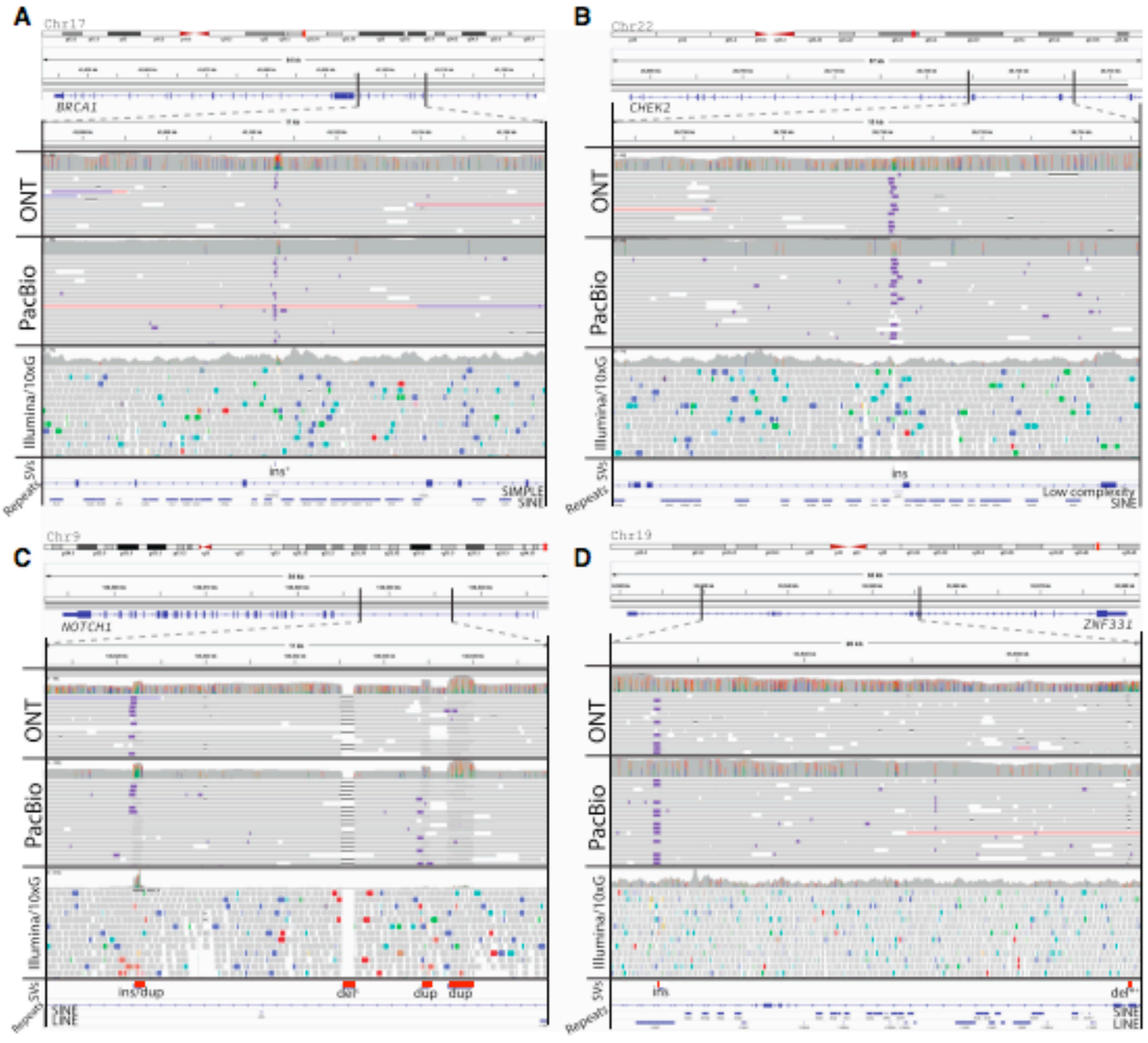
62bp repeat expansion in BRCA1 detected in normal tissue that is undetectable using a panel or short read sequencing

3,662 SVs specific to the tumor, most are undetectable using short read sequencing

	Total	Deletions	Duplications	Insertions	Inversions	Translocations
All SVs in normal	9816	5225	578	3727	130	156
All SVs in tumor	13737	7020	988	5292	202	235
SVs only in tumor (Also exclude NA12878)	3662	1805	420	1250	98	89

SVs in sample 51 not detected by short reads.

Insertions found in BRCA1 and CHEK2. Insertions and duplications found in NOTCH1.



GBE
Genome Biology and Evolution

JOURNAL ARTICLE

Long-Read Sequencing Reveals Rapid Evolution of Immunity- and Cancer-Related Genes in Bats

Armin Scheben, Olivia Mendivil Ramos, Melissa Kramer, Sara Goodwin, Sara Oppenheim, Daniel J Becker, Michael C Schatz, Nancy B Simmons, Adam Siepel ✉, W Richard McCombie ✉ Author Notes

Genome Biology and Evolution, Volume 15, Issue 9, September 2023, evad148,
https://doi.org/10.1093/gbe/evad148
Published: 20 September 2023 **Article history** ▾

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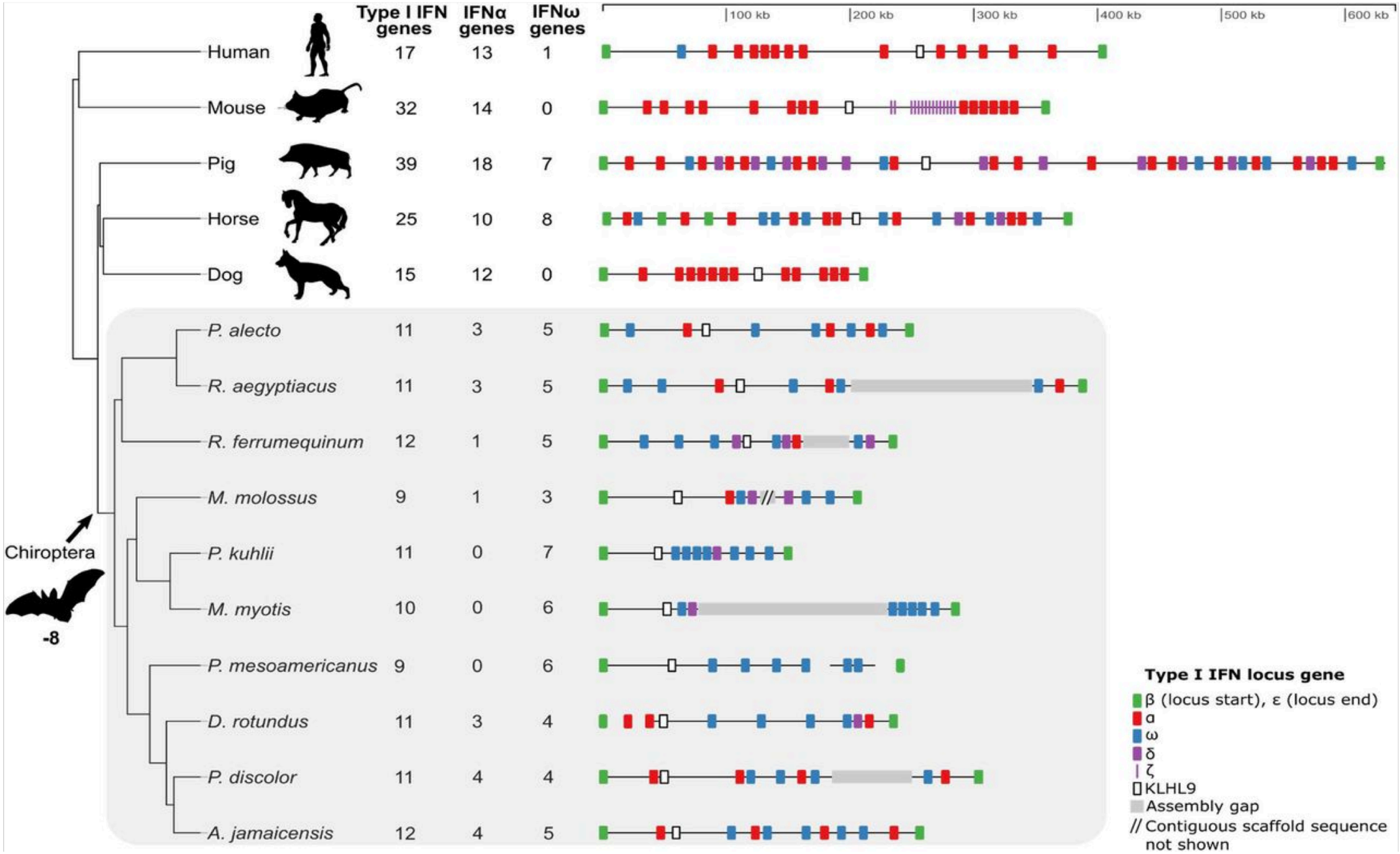
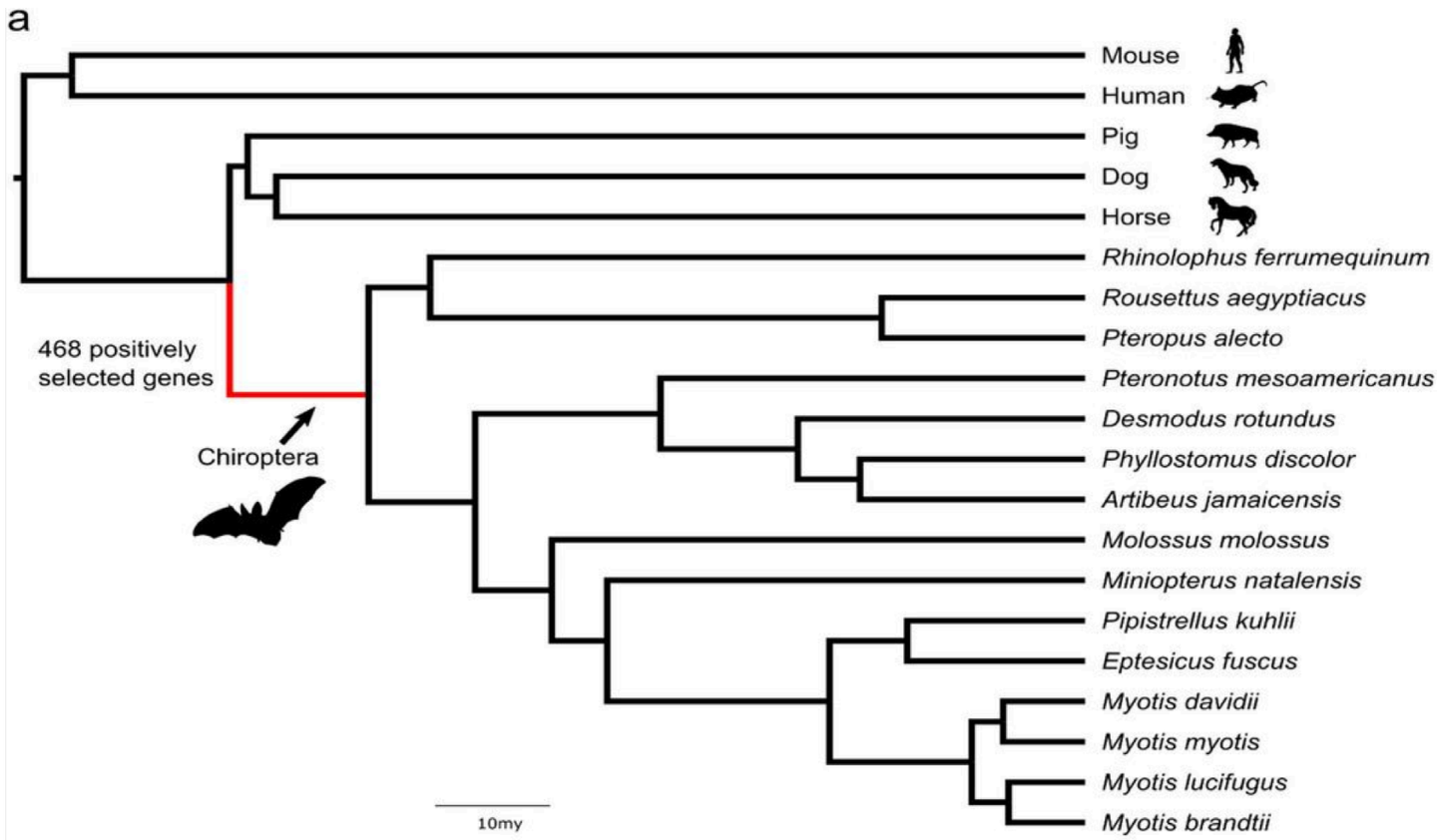
Abstract

Bats are exceptional among mammals for their powered flight, extended lifespans, and robust immune systems and therefore have been of particular interest in comparative genomics. Using the Oxford Nanopore Technologies long-read platform, we sequenced the genomes of two bat species with key phylogenetic positions, the Jamaican fruit bat (*Artibeus jamaicensis*) and the Mesoamerican mustached bat (*Pteronotus mesoamericanus*), and carried out a comprehensive comparative genomic analysis with a diverse collection of bats and other mammals. The high-quality, long-read genome assemblies revealed a contraction of interferon (IFN)-α at the immunity-related type I IFN locus in bats, resulting in a shift in relative IFN-ω and IFN-α copy numbers. Contradicting previous hypotheses of constitutive expression of IFN-α being a feature of the bat immune system, three bat species lost all IFN-α genes. This shift to IFN-ω could contribute to the increased viral tolerance that has made bats a common reservoir for viruses that can be transmitted to humans. Antiviral genes stimulated by type I IFNs also showed evidence of rapid evolution, including a lineage-specific duplication of IFN-induced transmembrane genes and positive selection in *IFIT2*. In addition, 33 tumor suppressors and 6 DNA-repair genes showed signs of positive selection, perhaps contributing to increased longevity and reduced cancer rates in bats. The robust immune systems of bats rely on both bat-wide and lineage-specific evolution in the immune gene repertoire, suggesting diverse immune

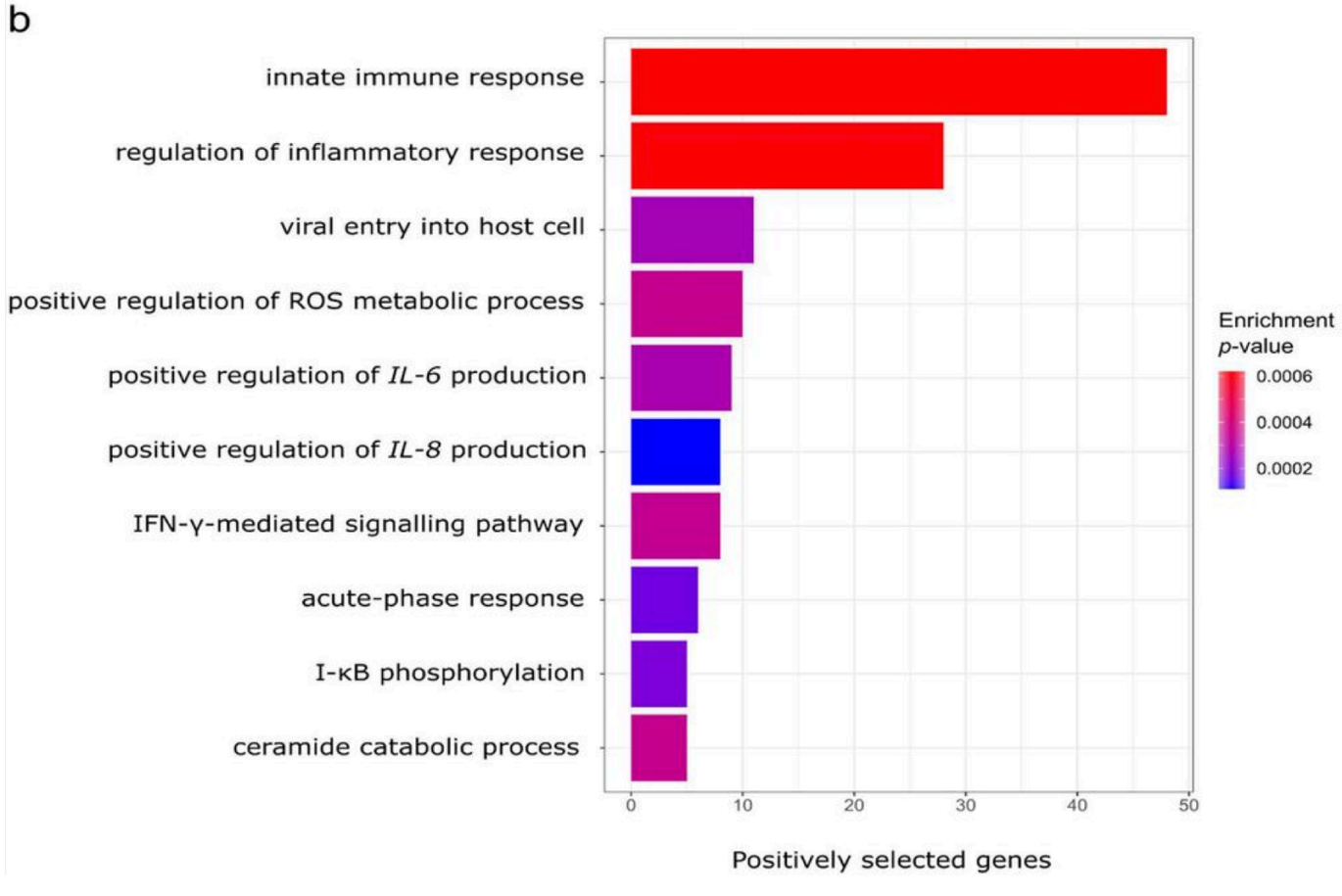
The type I Interferon (IFN) locus is contracted in bats compared to other mammals



Positive selection on the bat ancestral branch suggests strong enrichment of innate immunity genes



Eight of the ten most significant GO terms are related to immunity



DNA repair genes and tumor suppressors are positively selected in bats

Symbol	Name	aBSREL *P*-Value	Sites under Selection	Bat Branch ω	Outgroup Branch ω
CDH1	*Cadherin-1*	2.0e−6	3	0.17	0.29
CAT	*Catalase*	7.0e−6	1	0.38	>10
BIK	*BCL2 interacting killer*	3.7e−5	1	1.92	0.89
PALB2	*Partner and localizer of BRCA2*	1.8e−4	3	0.67	0.43
LATS2	*Large tumor suppressor kinase 2*	2.5e−4	10	0.23	0.11
SLC39A4	*Solute carrier family 39 member 4*	3.8e−4	3	0.55	0.42
SPARCL1	*SPARC like 1*	4.0e−4	4	0.88	0.95
PLA2G7	*Phospholipase A2 group VII*	8.5e−4	5	>10	1.70
GALR1	*Galanin receptor 1*	8.7e−4	1	0.10	0.08
CD79A	*CD79a molecule*	9.0e−4	1	>10	0.37
MYO1A	*Myosin IA*	9.8e−4	7	0.94	0.14

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Dear Abby: I want to bring my girlfriend to my son's wedding, do...

Gen Z and Gen Alpha are already beefing: 'We're gonna be made...

16 Comments

HEALTH

Bats could play vital role in preventing and treating cancer: 'first step' discovery

By Rob Bailey-Millado

Published Sep. 22, 2023, 12:28 p.m. ET

Bats get a deadly bad rap — but they could play a lifesaving role in cancer treatment, according to a "fascinating new study" that examines their remarkable immune systems.

As documented in peer-reviewed research in Genome Biology and Evolution, published Thursday by Oxford University Press, scientists from New York hope that by better understanding the winged creatures' extraordinary ability to both host and survive infections, they can develop ways to treat — and prevent — cancer in humans.

It might sound bat crazy, but these disease-riddled cave-dwelling creatures of the night might hold the key to curing CANCER

- **Bats are recognized for their ability to tolerate viruses and low rates of cancer**
- **READ MORE: Now the CDC wants to monitor your POOP to track flu outbreaks**

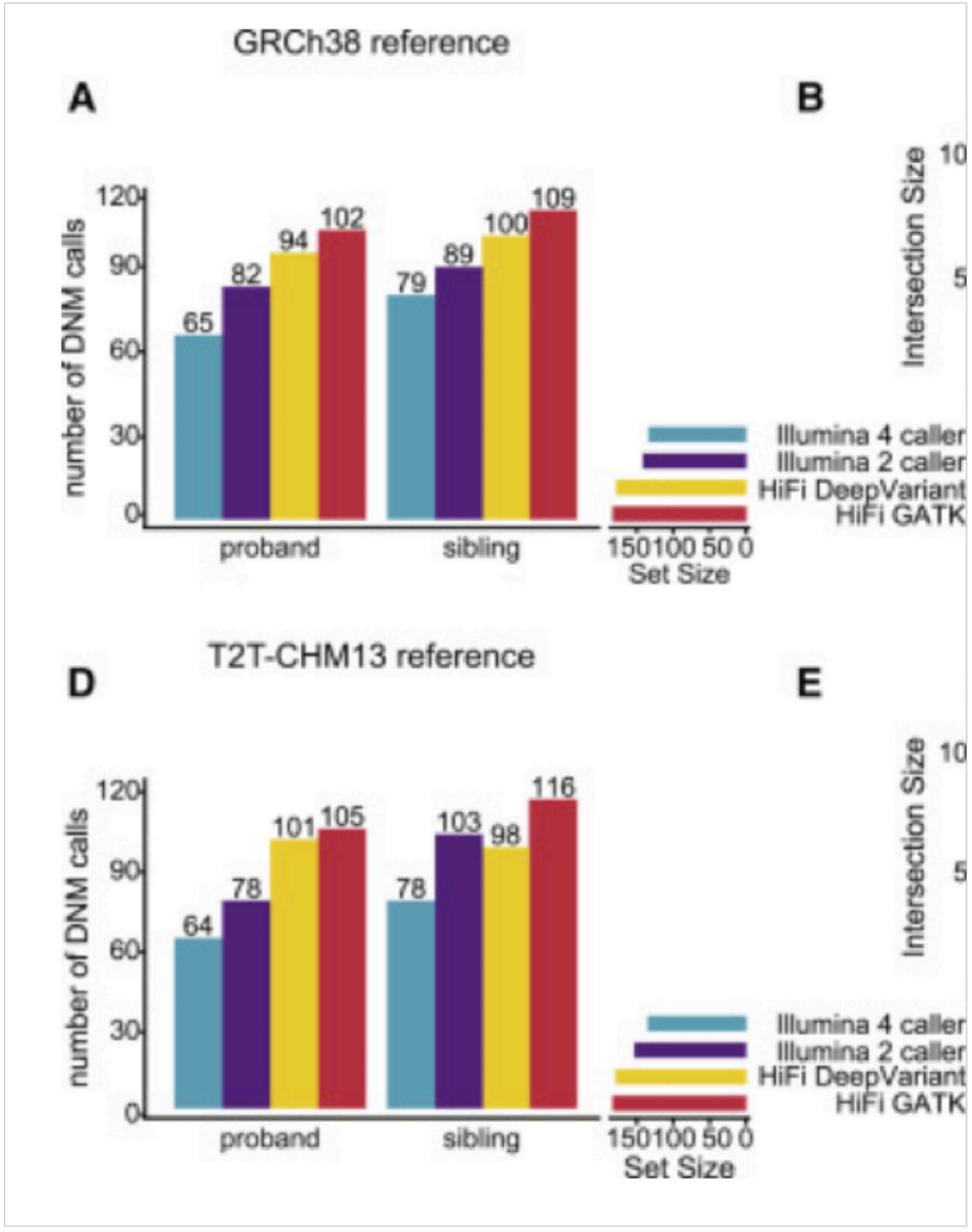
By BRITNEY NGUYEN FOR DAILYMAIL.COM

PUBLISHED: 07:31 EDT, 21 September 2023 | **UPDATED:** 08:47 EDT, 21 September 2023

Long read sequencing uncovers DNMs in Autism quad

Long reads, increased detection of De Novo variants including SNPs, InDels and SVs

Further improvement when mapping to the more complete CHM13 reference

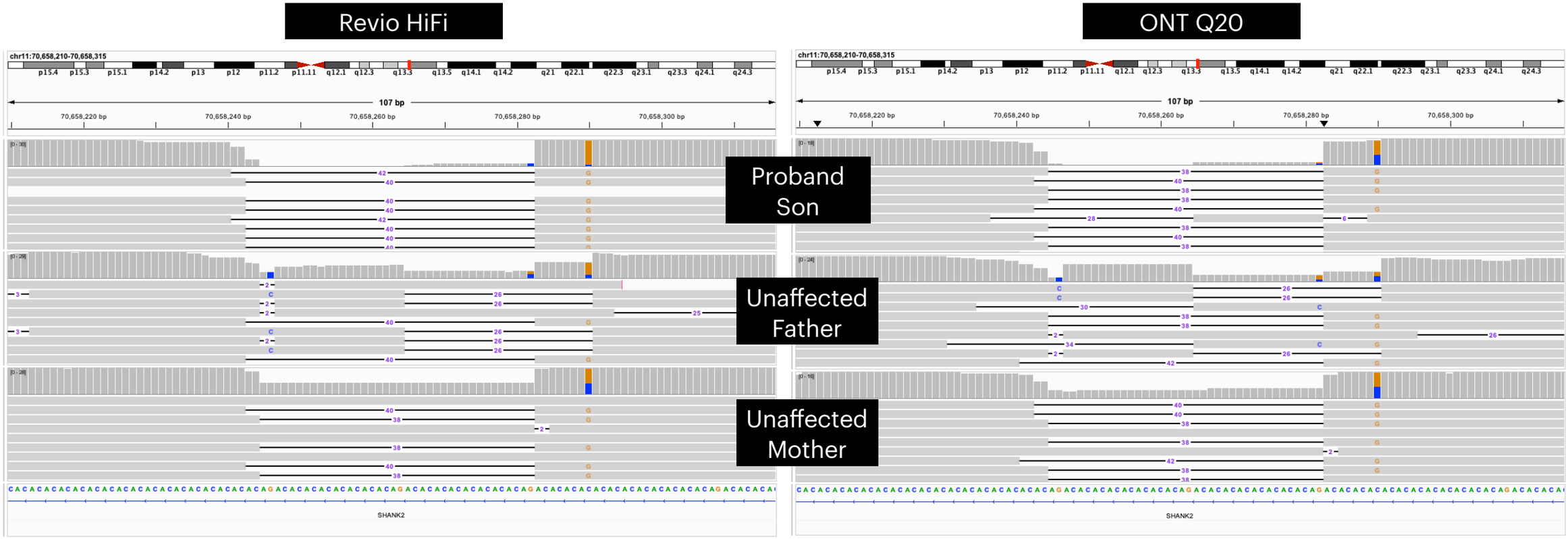


Michelle D. Noyes, William T. Harvey, David Porubsky, Arvis Sulovari, Ruiyang Li, Nicholas R. Rose, Peter A. Audano, Katherine M. Munson, Alexandra P. Lewis, Kendra Hoekzema, Tuomo Mantere, Tina A. Graves-Lindsay, Ashley D. Sanders, Sara Goodwin, Melissa Kramer, Younes Mokrab, Michael C. Zody, Alexander Hoischen, Jan O. Korbel, W. Richard McCombie, Evan E. Eichler

Familial long-read sequencing increases yield of de novo mutations

The American Journal of Human Genetics, Volume 109, Issue 4

Long Read Sequencing of Autism trio Cell Line DNA



Homozygous deletion in an intron of the gene SHANK2 , which has been associated with ASD

Long Read Germline Sequencing of Early Onset Cancer

SV Filtering Workflow

- Each individual will have ~25000 SV calls per genome
- Merge and genotype all calls across family members (~34000)
- Filter by family structure (~1400)
- Pull variants in/near genes (~450)
- Filter common events (~300)
- Filter false positives / ambiguous events/ select likely genes (~<100)

- No family history of cancer
- Standard IMPACT panel did not detect germline drivers

Cold Spring Harbor Laboratory

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Exploring the genetic and epigenetic underpinnings of early-onset cancers: Variant prioritization for long read whole genome sequencing from family cancer pedigrees

Melissa Kramer, Sara Goodwin, Robert Wappel, Matilde Borio, Kenneth Offit, Darren R. Feldman, Zsofia K. Stadler, W. Richard McCombie

doi: https://doi.org/10.1101/2024.06.27.601096

0 0 0 1 0 0 2

Abstract | Full Text | Info/History | Metrics | Preview PDF

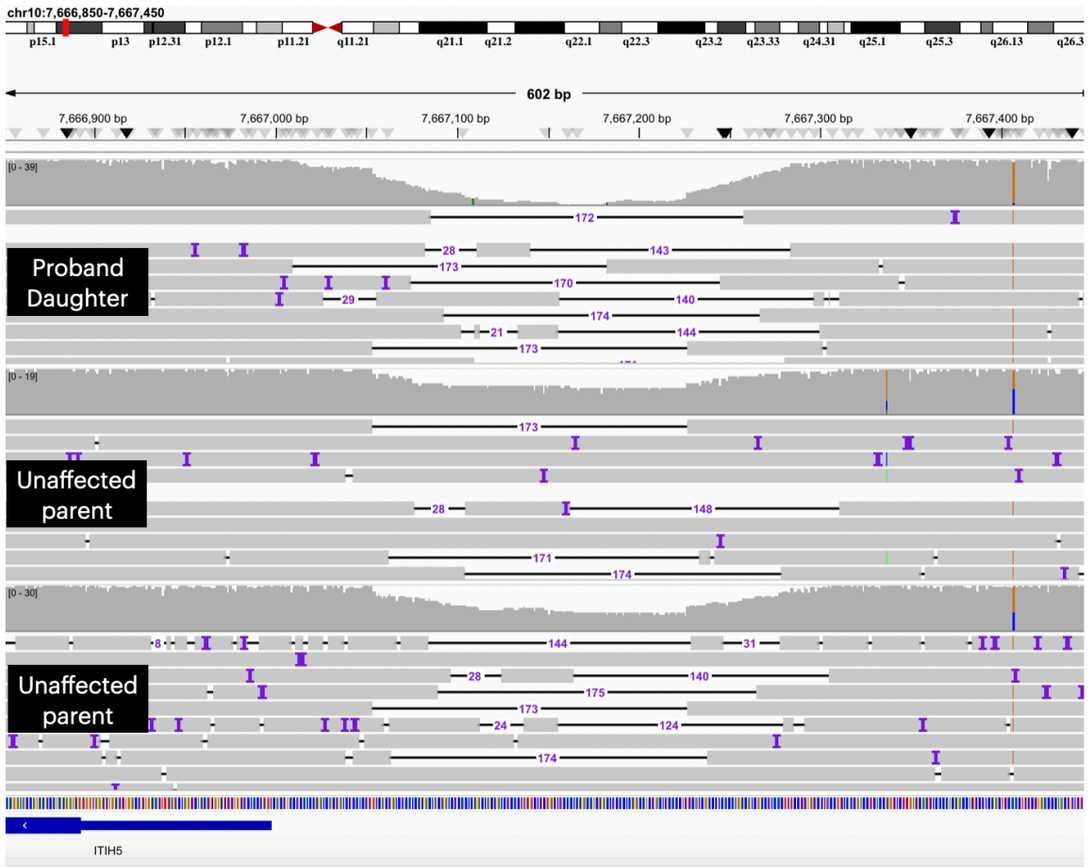
Abstract

Despite significant advances in our understanding of genetic cancer susceptibility, known inherited cancer predisposition syndromes explain at most 20% of early-onset cancers. As early-onset cancer prevalence continues to increase, the need to assess previously inaccessible areas of the human genome, harnessing a trio or quad family-based architecture for variant filtration, may reveal further insights into cancer susceptibility. To assess a broader spectrum of variation than can be ascertained by multi-gene panel sequencing, or even whole

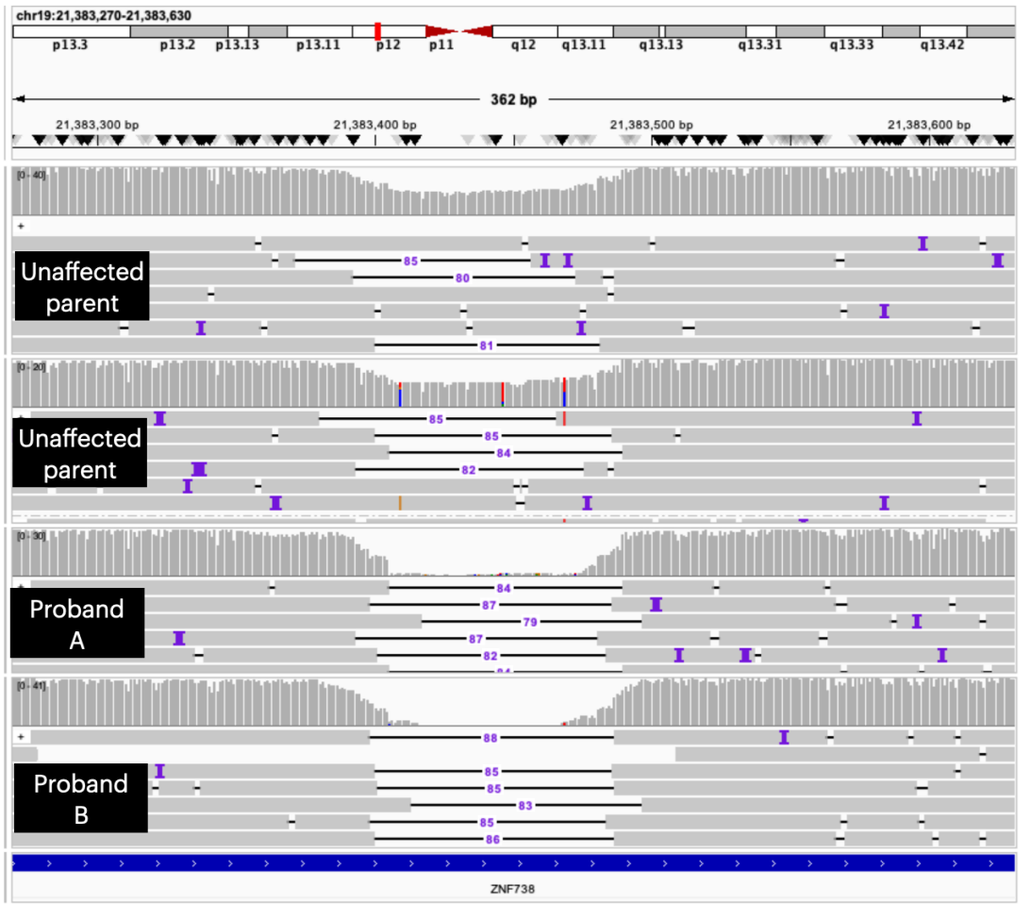
Collaboration with Zsofia Stadler MSKCC

Long Read Sequencing of Early Onset Cancer Pedigrees

Example of a structural variant which differs in the proband compared to the parents of EO-CRC trio #2, and overlaps a regulatory promoter region in ITIH5.

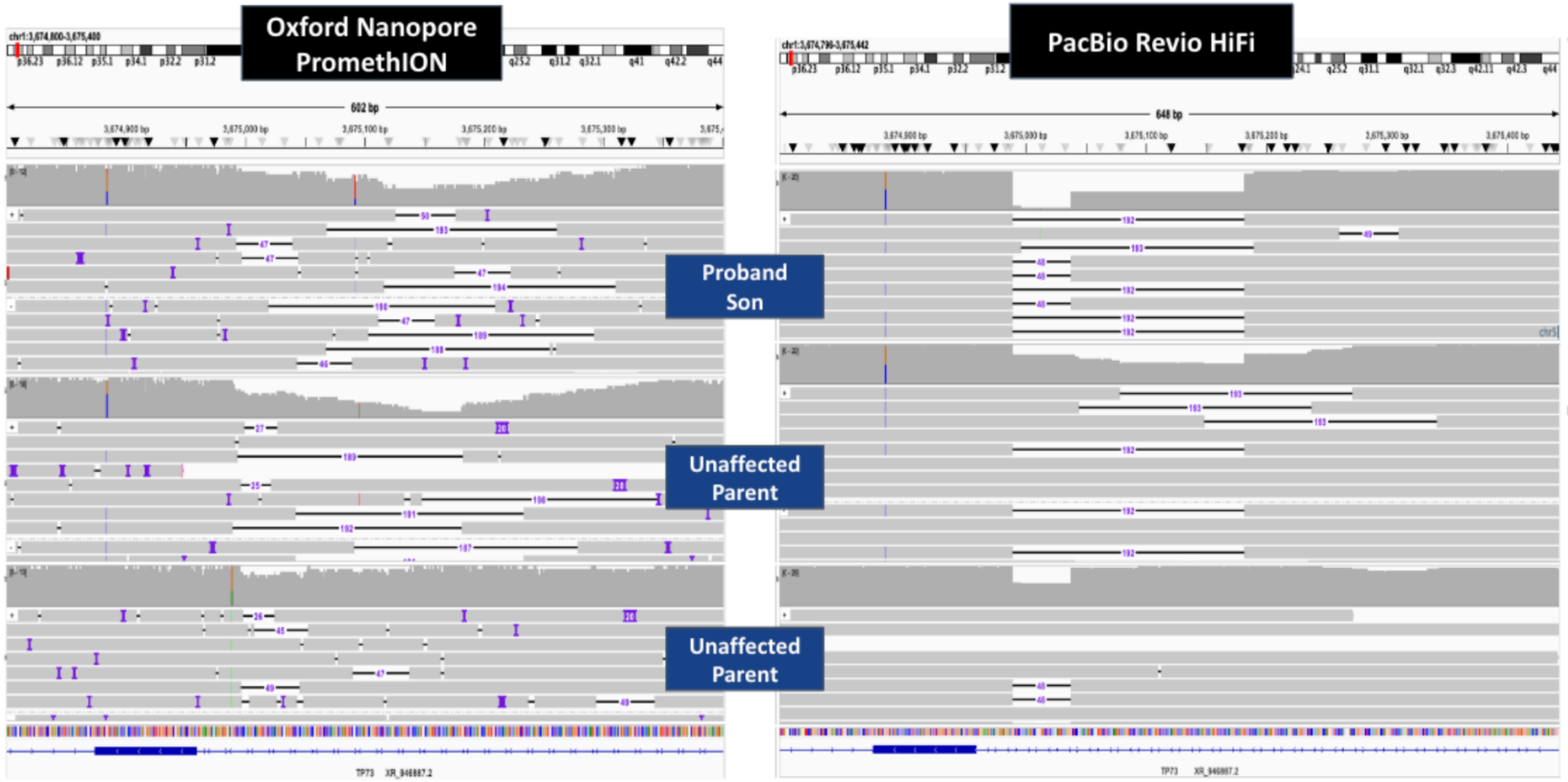


IGV screenshot of the last exon of gene ZNF738 in the TGCT quad. Aligned reads are displayed with heterozygous deletions in the unaffected parents (top panels), but homozygous deletions in both probands (bottom panels).



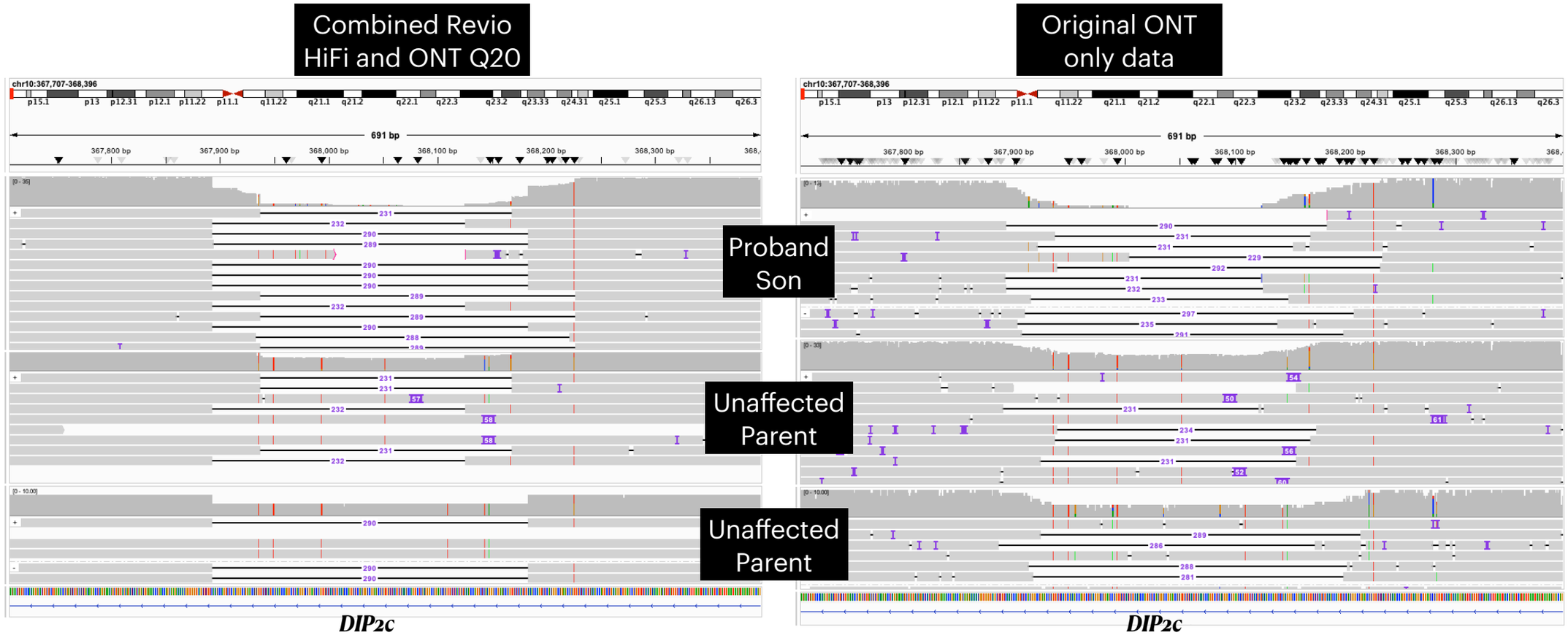
Collaboration with Zsofia Stadler MSKCC

PacBio Revio sequencing improves resolution of breakpoints and haplotyping of neighboring SNVs



Comparison of original ONT sequencing versus PacBio HiFi for structural variant in gene *TP73*. The proband harbors deletions on both alleles, while each parent has one allele deleted, either a 48bp deletion or a 192bp deletion.

Combination of ONT and PacBio sequencing improves resolution, decreases cost and takes advantage of long ONT read lengths with HiFi accuracy

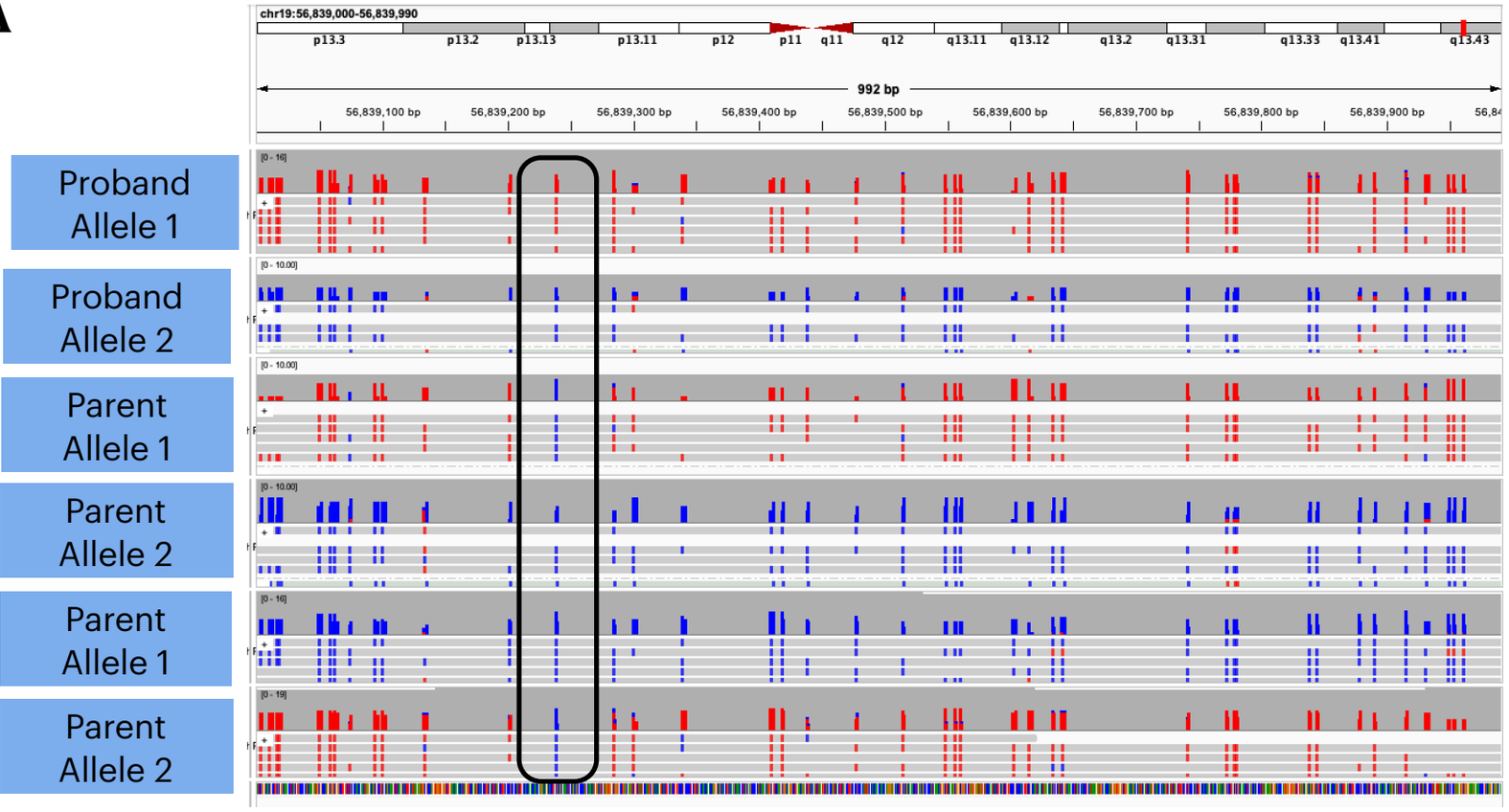


IGV screenshot of deleted intronic region of *DIP2C* using updated Revio HiFi sequencing combined with ONT Q20 data versus the older ONT chemistry. Each unaffected parent has one deleted allele while the proband harbors both deleted alleles.

Collaboration with Zsofia Stadler MSKCC

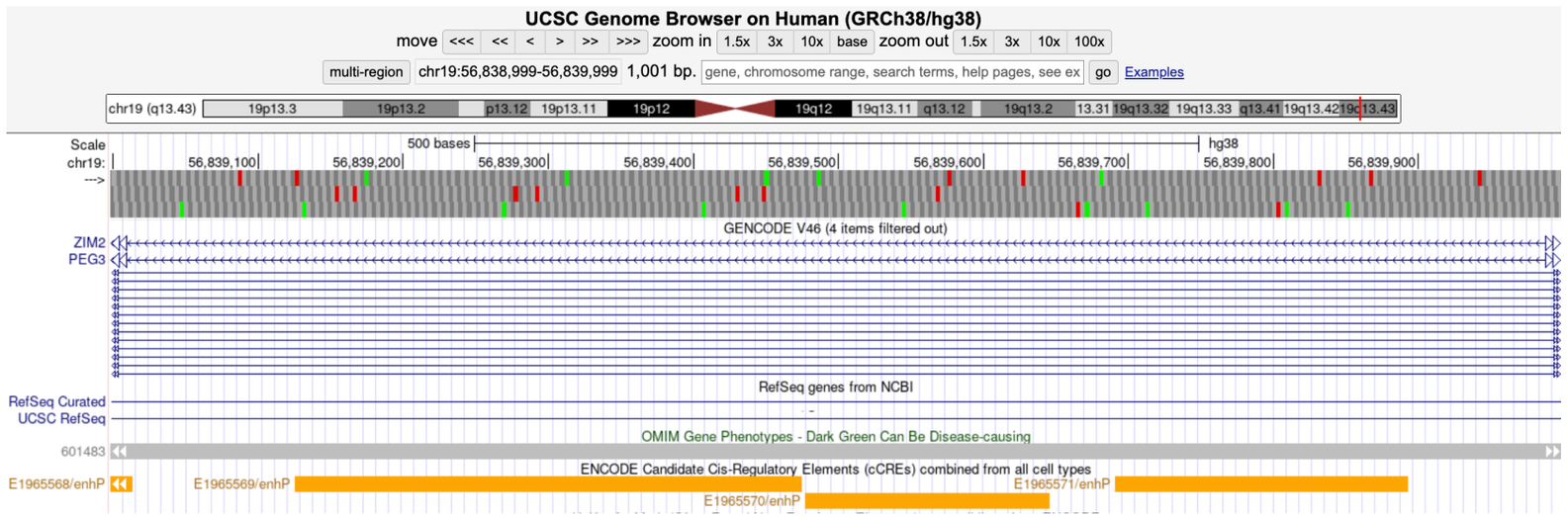
Phasing methylation provides allele specific context for PEG3 in EO-CRC trio #2

A



IGV screenshot showing phased alleles of the proband and parents. In “bisulfite mode”, red colored bases represent methylated Cs and blue indicates unmethylated Cs. Each family member (parents and proband) contains one methylated (silenced) allele in red, and one expressed allele. Black encircled CpG site is unmethylated in all parental alleles but methylated in one proband allele.

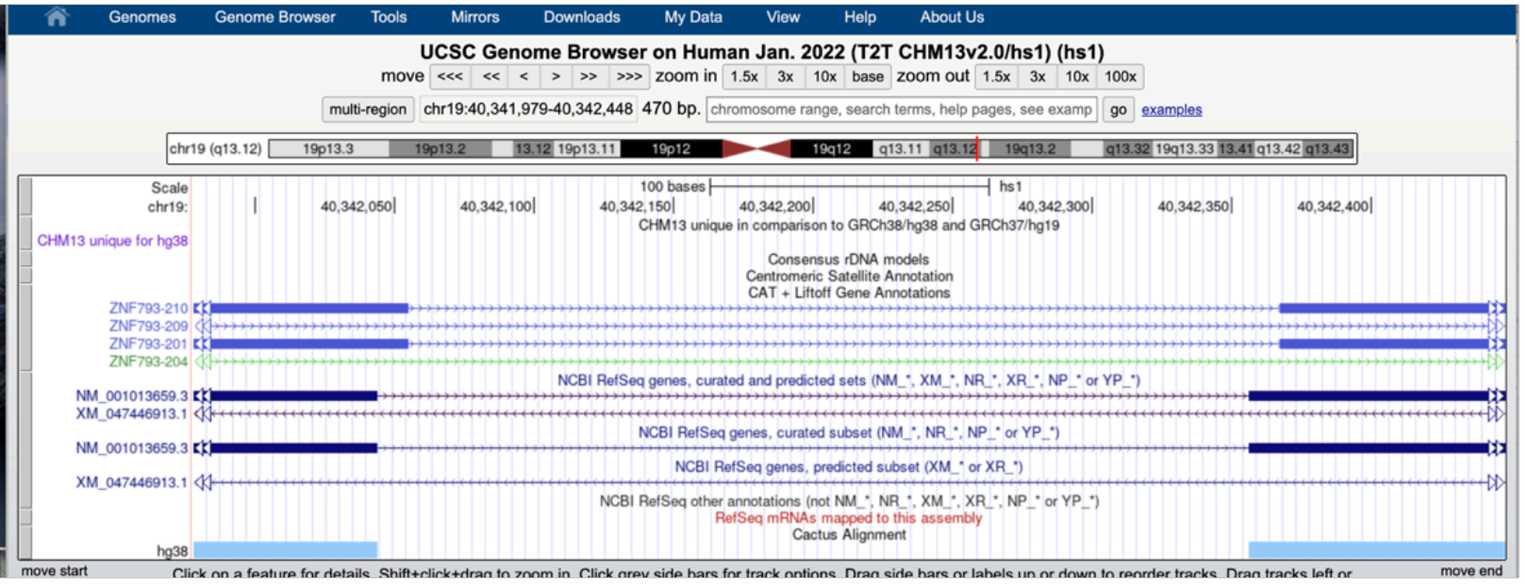
B



UCSC Genome browser showing overlap with ENCODE enhancer marks.

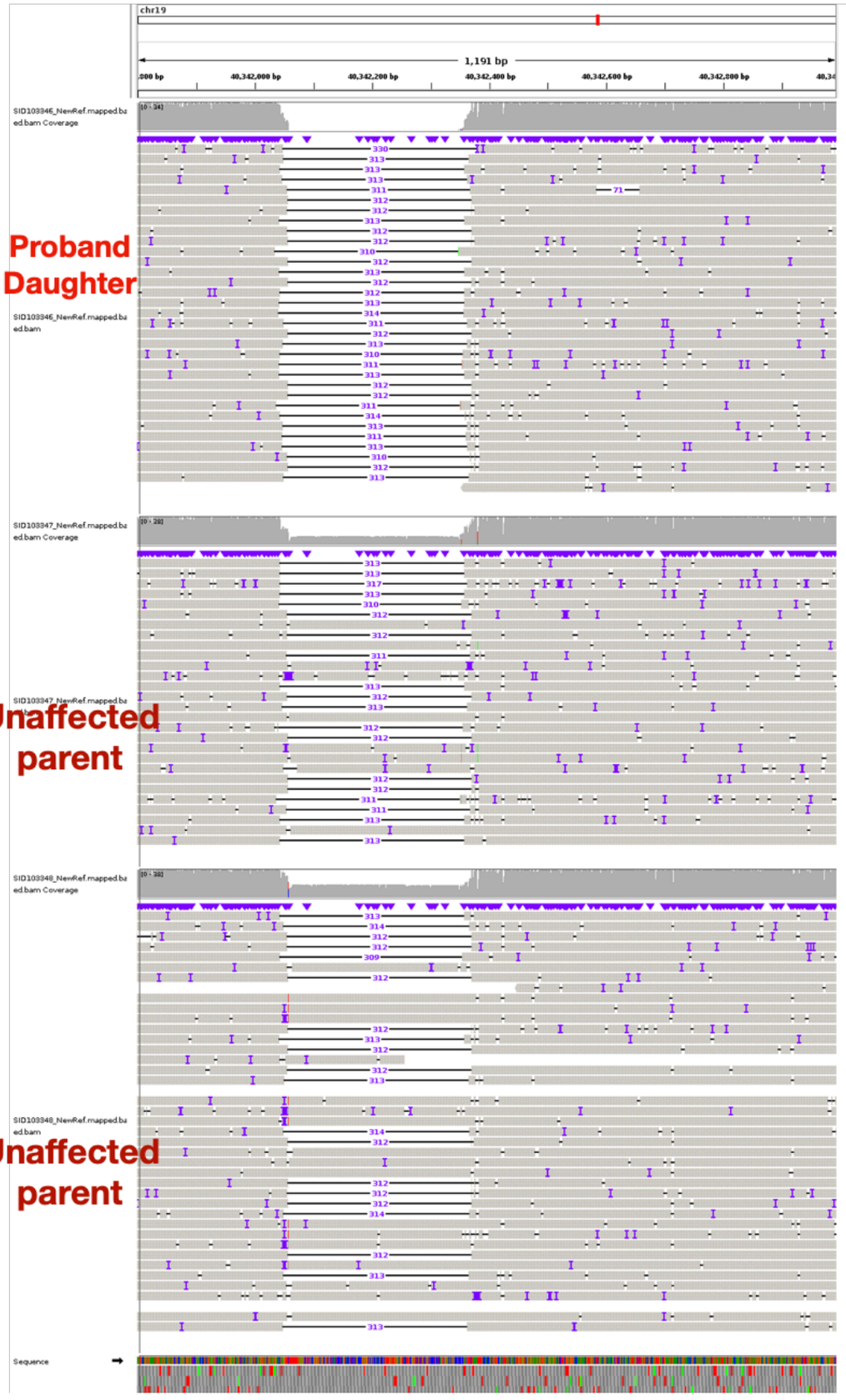
Improved T2T reference genome uncovers new variants

ZNF793 deletion overlaps last exon
Has been associated with lung cancer and Barrett's esophagus



Break in the alignment of CHM13 compared to hg38 in this region

Colon cancer trio 2



Nanopore error rates are improving

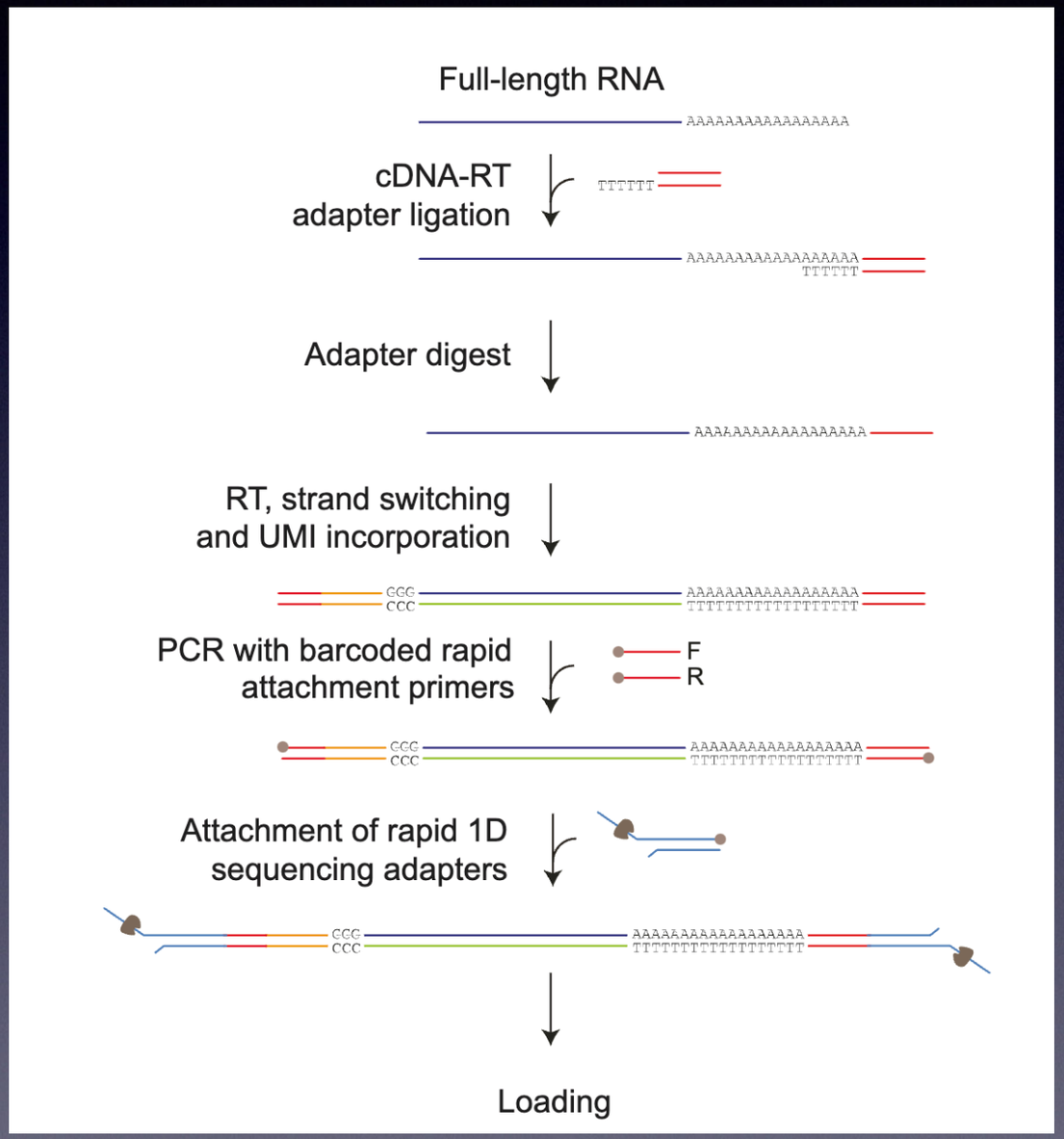
Chemistry and software improvements are increasing accuracy

ONT simplex reads with Dorado (single read):
~1% error (Q20)

ONT now recommends HERRO read correction to drive quality to ~Q30-Q40

Transcriptome Sequencing on Oxford Nanopore

ONT PCR cDNA



Low input (~1ng poly A+)

PCR may introduce biases

Enriched for full length cDNA (template switching)

Multiplex up to 96 samples

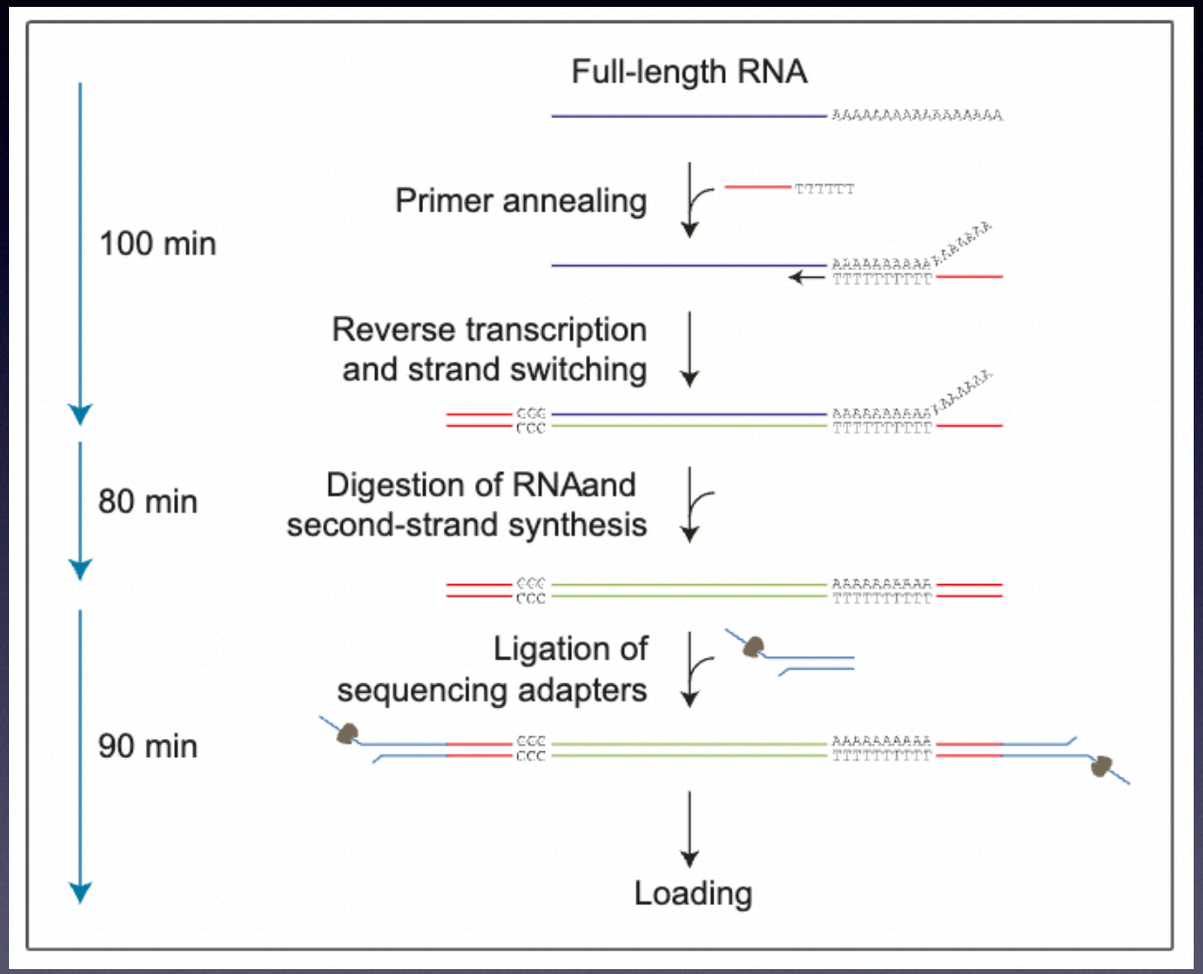
Much higher throughput (>90 million reads per PromethION cell, up to ~200M)

Lengths ~700bp

Recent paper shows 40 fold fewer long reads (8 fold fewer bases) are required to cover 6000 genes across 95%

Oikonomopoulos S, Bayega A, Fahiminiya S, Djambazian H, Berube P, Ragoussis J. Methodologies for Transcript Profiling Using Long-Read Technologies. Front Genet. 2020 Jul 7;11:606.

ONT direct cDNA



Requires more input (~50 poly A+)

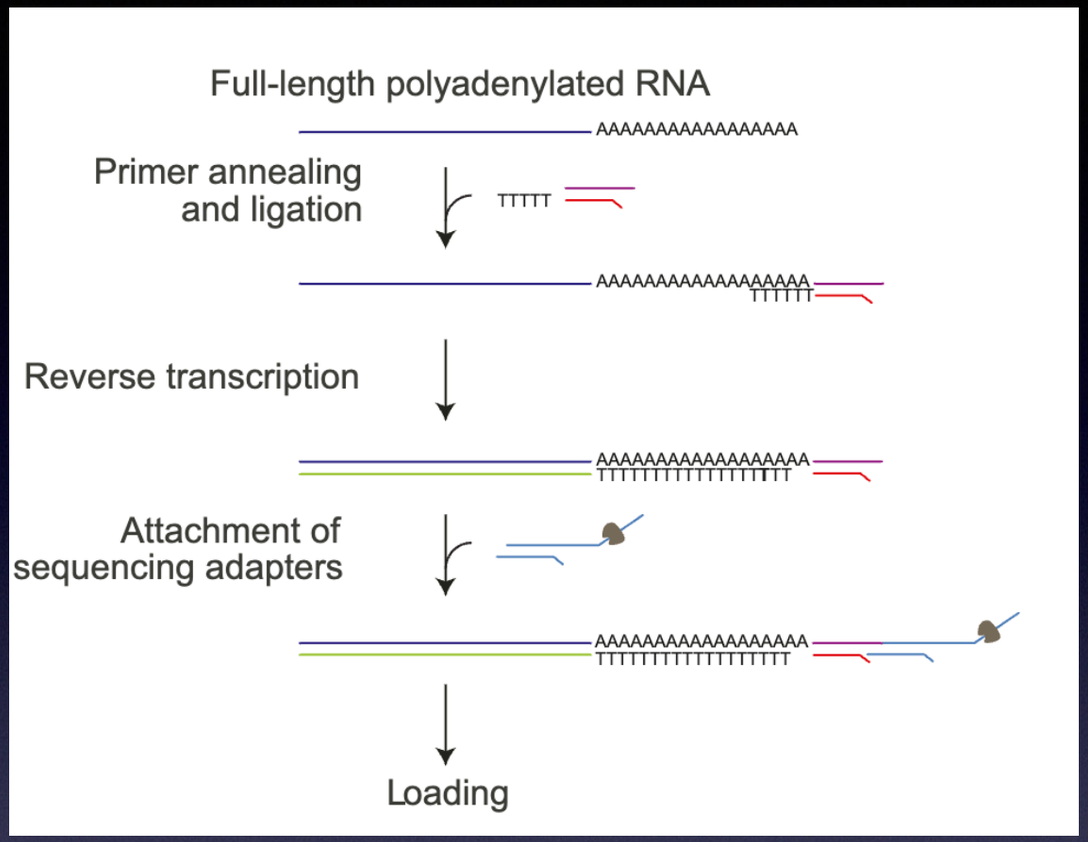
Does not use PCR

Enriched for full length cDNA (template switching)

Can multiplex with native barcoding kit

Less throughput (40-50 million reads per PromethION cell), lengths a bit longer ~1.5kb

ONT direct RNA



Many tools have been/are being developed to use the raw ONT signal data to detect modifications

Tombo (Stoiber et al 2017)
Nanocompore (Leger et al 2021)
xPore (Pratanwanich et al 2021)
nanoRMS (Begik et al 2021)

Input requirement (100 ng poly-A^+ RNA, though total RNA can be used with caveats)

RNA length preserved

No PCR, RT is optional

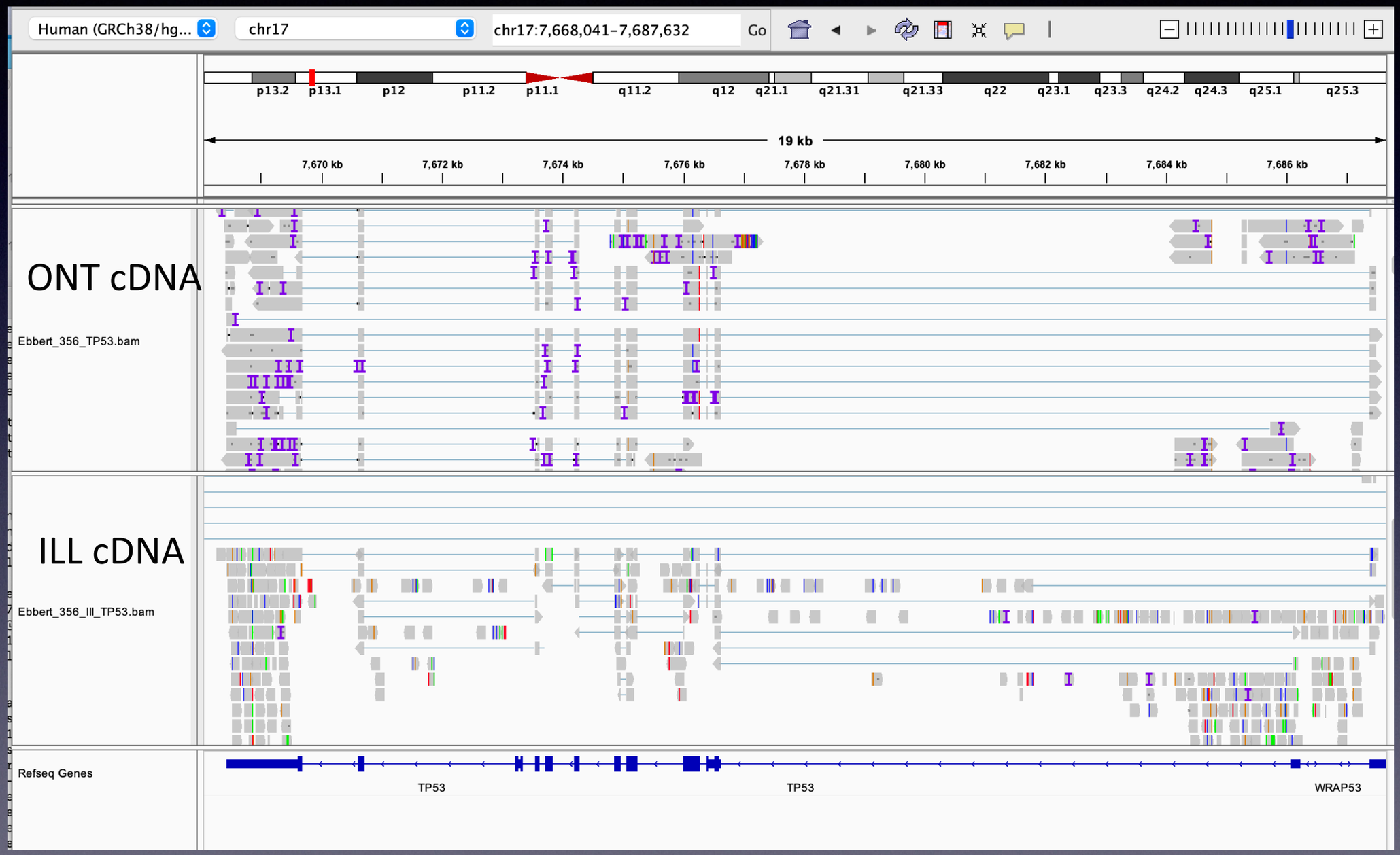
Can detect base modifications (6mA, 5mC)

Output is much lower, ~20 million reads on PromethION RNA specific cels

Lengths 1.5-2kb

Ribosomal RNA depletion is an issue

Long reads span junctions and provide connection



Transcript Coverage

StringTie2 (Kovaka 2019) used to assemble transcripts and detect genes where transcripts were fully covered end to end by reads

Illumina data - 16,476 genes

ONT full data set - 25,478 genes

ONT 50pct downsample - 21,322 genes

ONT 75pct downsample - 19,088 genes

Transcriptome Sequencing Cost Comparisons Across Platforms

Illumina cost per million reads
$6

ONT PCR cDNA cost per million reads
$10

PacBio IsoSeq cost per million reads
$20

Tradeoffs on accuracy and length, so it is key to assess the method that will address critical questions of your experiment

Gapless assembly of complete human and plant chromosomes using only nanopore sequencing

Sergey Koren, Zhigui Bao, Andrea Guarracino, Shujun Ou, Sara Goodwin, Katharine M. Jenike, Julian Lucas, Brandy McNulty, Jimin Park, Mikko Rautiainen, Arang Rhie, Dick Roelofs, Harrie Schneiders, Ilse Vrijenhoek, Koen Nijbroek, Doreen Ware, Michael C. Schatz, Erik Garrison, Sanwen Huang, W. Richard McCombie, Karen H. Miga, Alexander H.J. Wittenberg, Adam M. Phillippy

doi: https://doi.org/10.1101/2024.03.15.585294

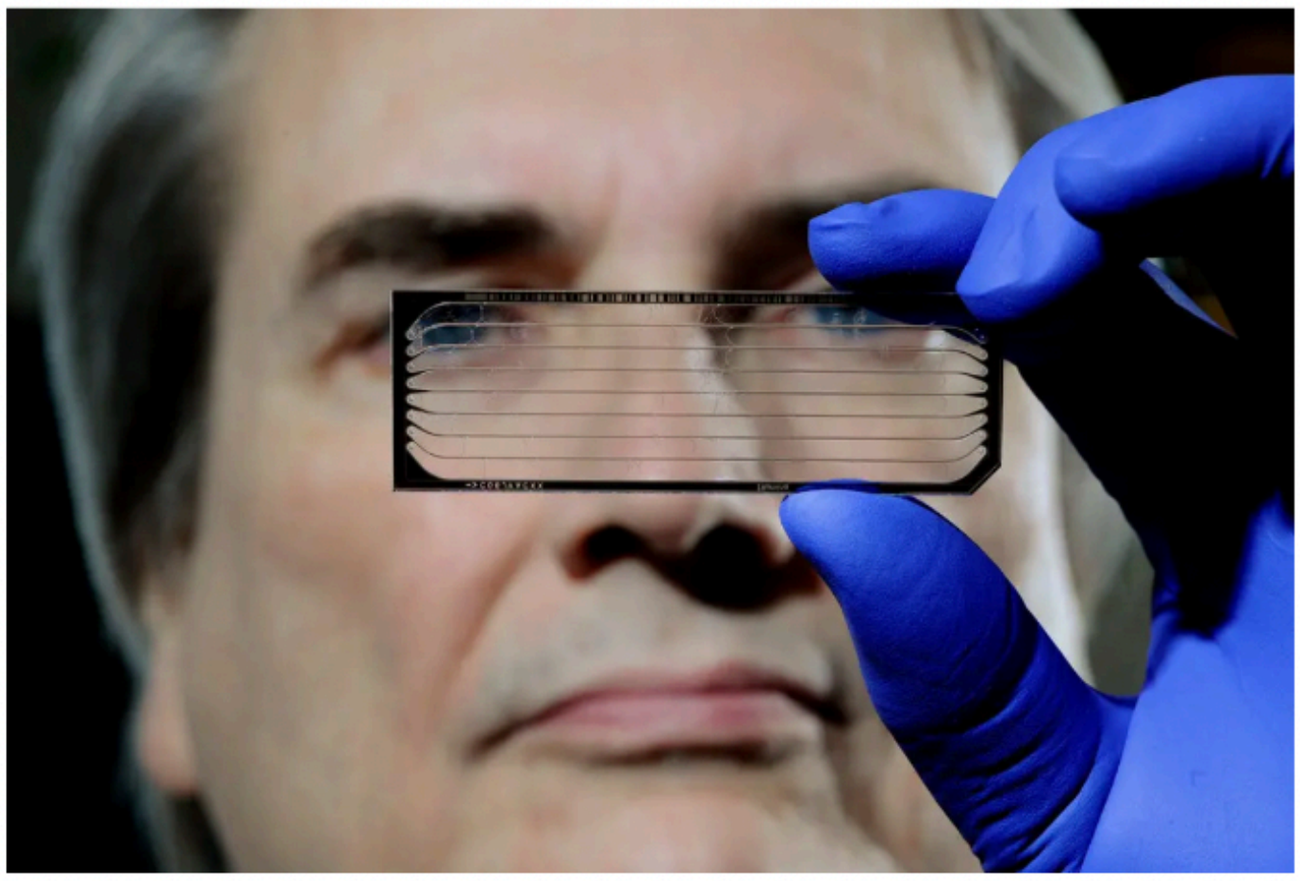
Posted March 19, 2024.

This article is a preprint and has not been certified by peer review [what does this mean?].

Subject Area

Bioinformatics

DNA Sequencing Caught in Deluge of Data



W. Richard McCombie, a professor of human genetics at the Cold Spring Harbor Laboratory, examining DNA samples. Kathy Kmonicek for The New York Times

Conclusions

- Substantial variation is missed by short reads
- Phasing can reveal more detailed context of variation
- Massive data storage problems are worse for long read sequencing
- Interpretation of SVs must be optimized to understand regulatory effects of widespread variation

Summary

Long read platforms have matured significantly in the last few years

PacBio and Oxford Nanopore producing similar length distributions

Overcome high error sequencing with improved informatics

Oxford Nanopore exciting for methylation & direct RNA capabilities

Long reads are crucial for accurate SV calling

Finding thousands to tens of thousands of additional SVs over short reads

Resolves the false positives observed with short reads

Detecting potential cancer risk factors that would otherwise go unnoticed

Sample & DNA requirements one of the largest barriers for clinical application

Continue to advance protocols for extracting, preparing samples

Organoids (as opposed to primary tumors) enable large DNA amounts for long read sequencing, though it remains much more difficult then cell culture

Organoids also enable application and profiling of other molecular and pharmaceutical assays

Future goals

Reduce sample DNA input - tumors, single cell, targeting - Shruti Iyer

Analyze data from projects for relevant genome properties

Improve long read sequencing efficiency - read length, yield, combination of input data types

Optimum cost benefit analyses of different long read approaches and coverage

Optimize long read transcriptome sequencing

Acknowledgements

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Spector Lab

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Schatz Lab

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Timp Lab

Isac Lee

AMNH

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