Bioinformatics Workshop Agenda – June 27, 2017

Hosted by the Genomics Resource Center,
University of Maryland School of Medicine

THE LEADER IN LONG-READ SEQUENCING

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8:30 – 8:55 a.m.	**Registration and Continental Breakfast**
9:00 – 9:10 a.m.	Welcome and Introduction Roberto Lleras, Manager, Field Applications Scientist, Bioinformatics, PacBio
9:10 – 9:55 a.m.	**Introduction to SMRTLink 5.0**
	Minor Variant Detection with Juliet Roberto Lleras, Manager, Field Applications Scientist, Bioinformatics, PacBio
	Structural Variant Detection with PBSV Aaron Wenger, Ph.D., Staff Scientist, PacBio
10:00 – 11:15 a.m.	**Concurrent Breakout Sessions**
	SESSION I: So, I Have a Diploid Assembly…Now What?
10:00 – 10:30 a.m.	Understanding, Curating, and Analyzing Your Diploid Genome Assembly Sarah Kingan, Ph.D., Senior Scientist, Bioinformatics, PacBio
10:30 – 10:45 a.m.	Chromosome-scale De Novo Assembly of Mammalian Genomes Using Chromatin Interaction Data Jay Ghurye, Ph.D. Candidate, Department of Computer Science, University of Maryland
10:45 – 11:15 a.m.	SESSION I Discussion
	SESSION II: Mini-training Session: Best Practices in Multiplexing with PacBio
10:00 – 10:30 a.m.	Best Practice for Interpreting Demultiplexed Output Carmen Guarco, Ph.D., Scientist, Field Applications Support, Bioinformatics, PacBio
10:30 – 10:50 a.m.	Downstream Applications: Minor Variant Calling, Microbial Assembly, CCS2, and Iso-Seq Roberto Lleras, Manager, Field Applications Scientist, Bioinformatics, PacBio
10:50 – 11:15 a.m.	SESSION II Discussion
11:15 – 11:30 a.m.	**Coffee Break**
11:30 – 11:45 a.m.	Breakout Sessions Wrap-up
11:45 – 12:30 p.m.	Keynote: Accurate Detection of Complex Structural Variation Fritz J. Sedlazeck, Ph.D., Lead Scientific Programmer, Human Genome Sequencing Center, Baylor College of Medicine
12:30 – 12:45 p.m.	Open Forum for User Questions, Comments and Feedback on SMRTLink
12:45 – 12:55 p.m.	Closing Remarks
1:00 p.m.	**Lunch**

Thanks to our Partners:

Understanding, Curating, and Analyzing your Diploid Genome Assembly

Sarah B. Kingan, Ph.D.
Senior Scientist, Bioinformatics, PacBio Applications

East Coast UGM, Baltimore, MD
Tuesday June 27th 2017

AGENDA

- **Understanding Your Diploid Assembly**
  - Assembly Workflow
  - Heterozygosity and Coverage
- **Curating your Assembly**
  - Filtering Contigs
  - Deduplicating Haplotypes
- **Submitting Your Assembly to NCBI**

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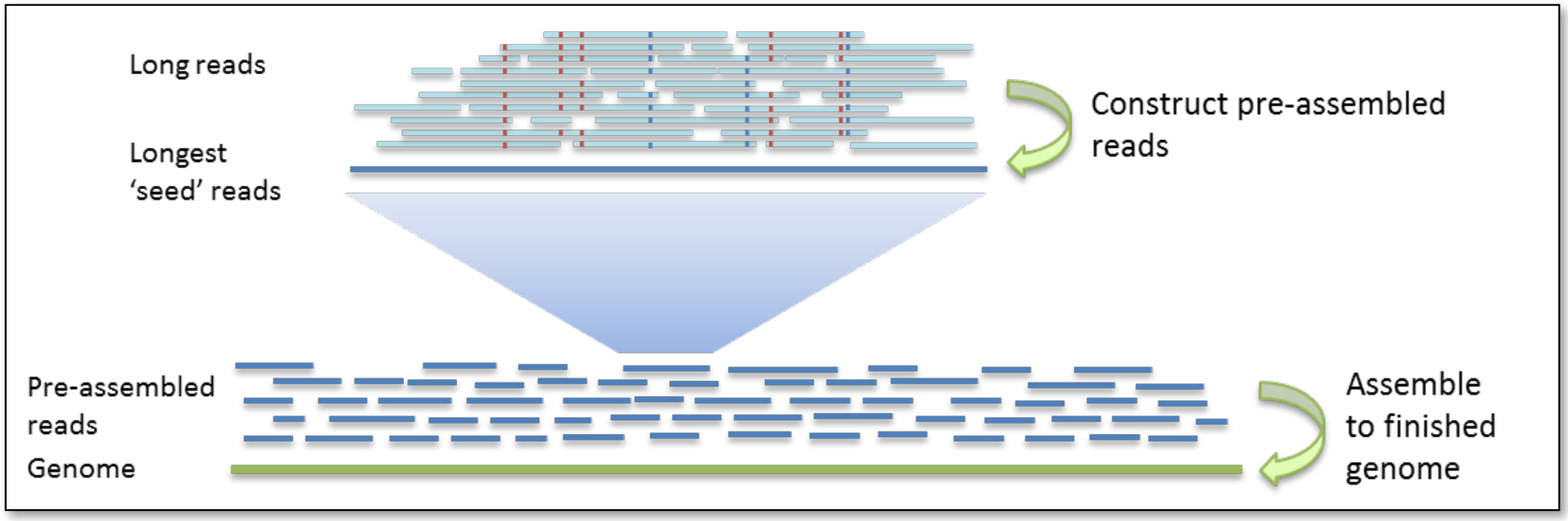
Understanding Your Diploid Assembly

Assembly Workflow: From Raw Reads to High Quality Reference

FALCON / HIERARCHICAL GENOME ASSEMBLY PROCESS (HGAP)

RAW READS → PREASSEMBLED READS → CONTIG ASSEMBLY

"ERROR-CORRECTED" READS



Chin, et al. (2013). Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. Nature Methods. 10(6), 563.

FALCON AND FALCON-UNZIP

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Phased diploid genome assembly with single-molecule real-time sequencing.

Chin CS, Peluso P, Sedlazeck FJ, Nattestad M, Concepcion GT, Clum A, Dunn C, O'Malley R, Figueroa-Balderas R, Morales-Cruz A, Cramer GR, Delledonne M, Luo C, Ecker JR, Cantu D, Rank DR, Schatz MC

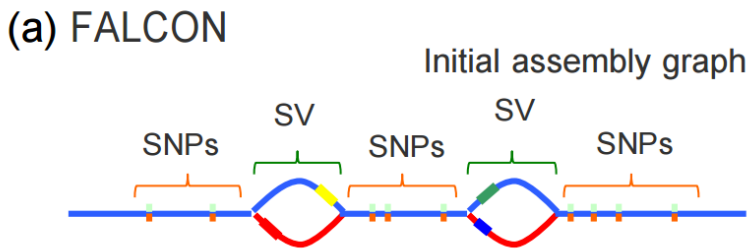
ABSTRACT

While genome assembly projects have been successful in many haploid and inbred species, the assembly of noninbred or rearranged heterozygous genomes remains a major challenge. To address this challenge, we introduce the open-source FALCON and FALCON-Unzip algorithms (https://github.com/PacificBiosciences/FALCON/) to assemble long-read sequencing data into highly accurate, contiguous, and correctly phased diploid genomes. We generate new reference sequences for heterozygous samples including an F1 hybrid of Arabidopsis thaliana, the widely cultivated Vitis vinifera cv. Cabernet Sauvignon, and the coral fungus Clavicorona pyxidata, samples that have challenged short-read assembly approaches. The FALCON-based assemblies are substantially more contiguous and complete than alternate short- or long-read approaches. The phased diploid assembly enabled the study of haplotype structure and heterozygosities between homologous chromosomes, including the identification of widespread heterozygous structural variation within coding sequences.

- FALCON is a **diploid-aware assembler.**
- FALCON-Unzip module performs true **phased assembly** for diploid samples.

Chin et al. (2016). Phased diploid genome assembly with single-molecule real-time sequencing. Nature Methods. 13(12), 1050.

DIPLOID ASSEMBLY WITH FALCON-UNZIP



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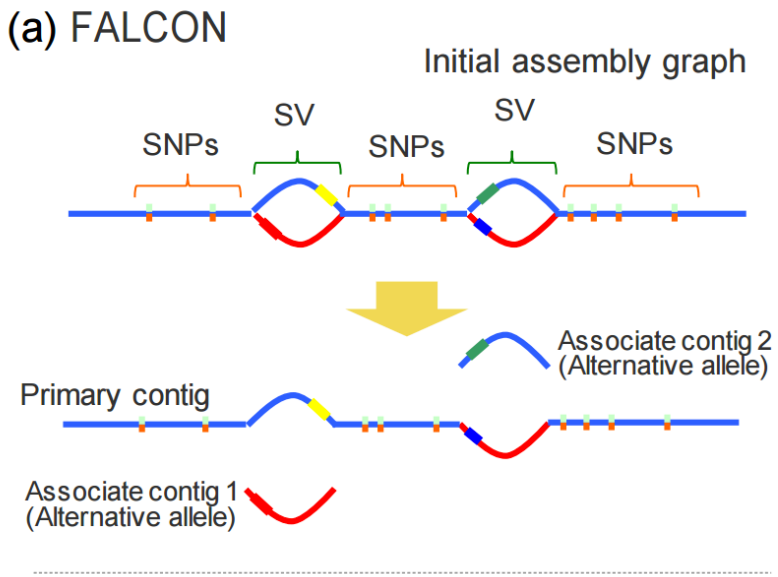

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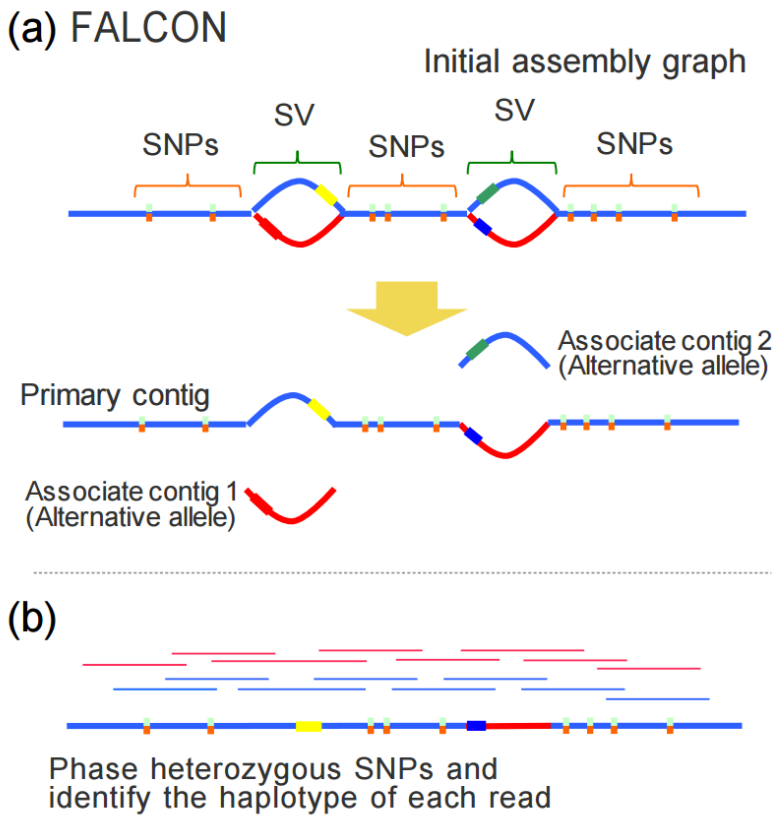

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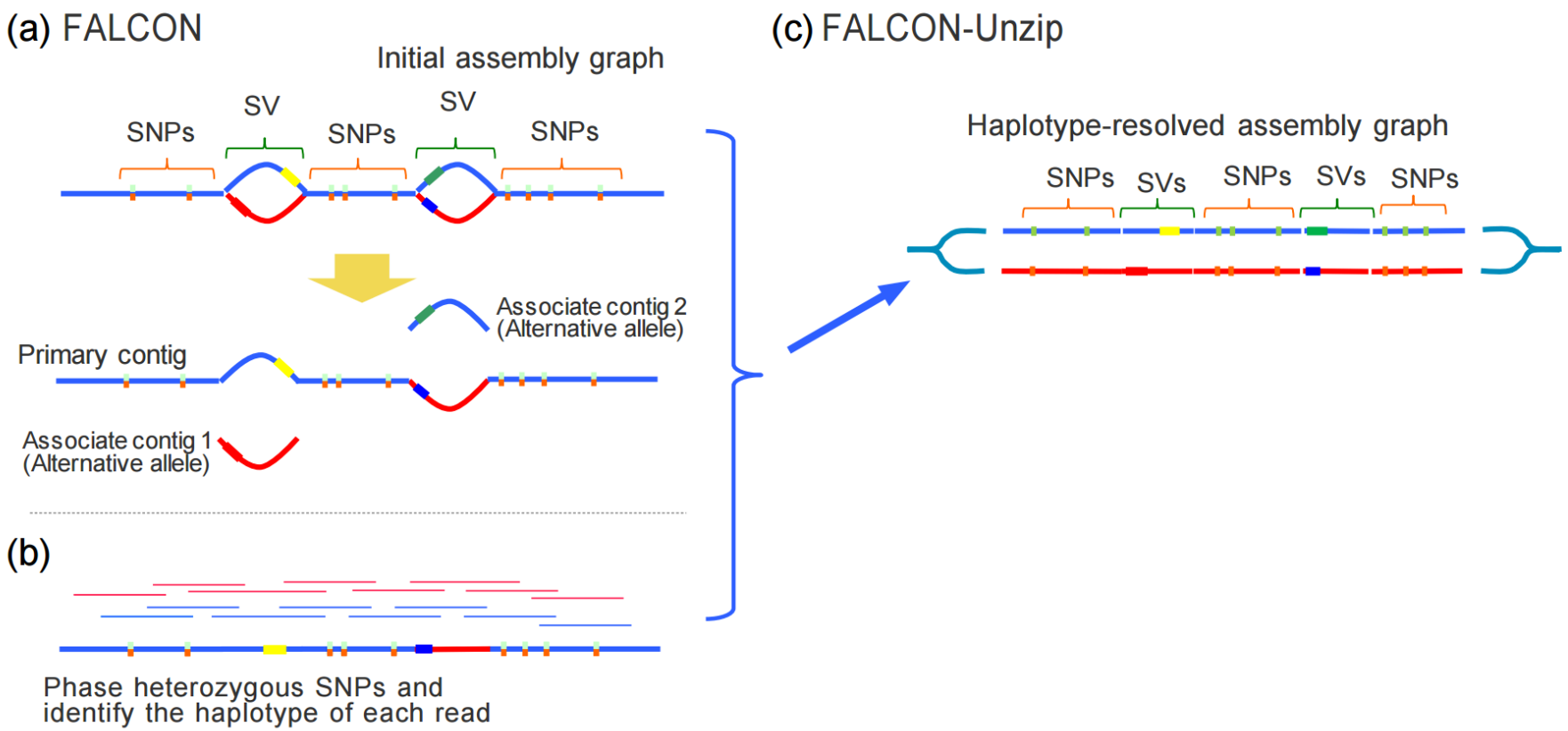
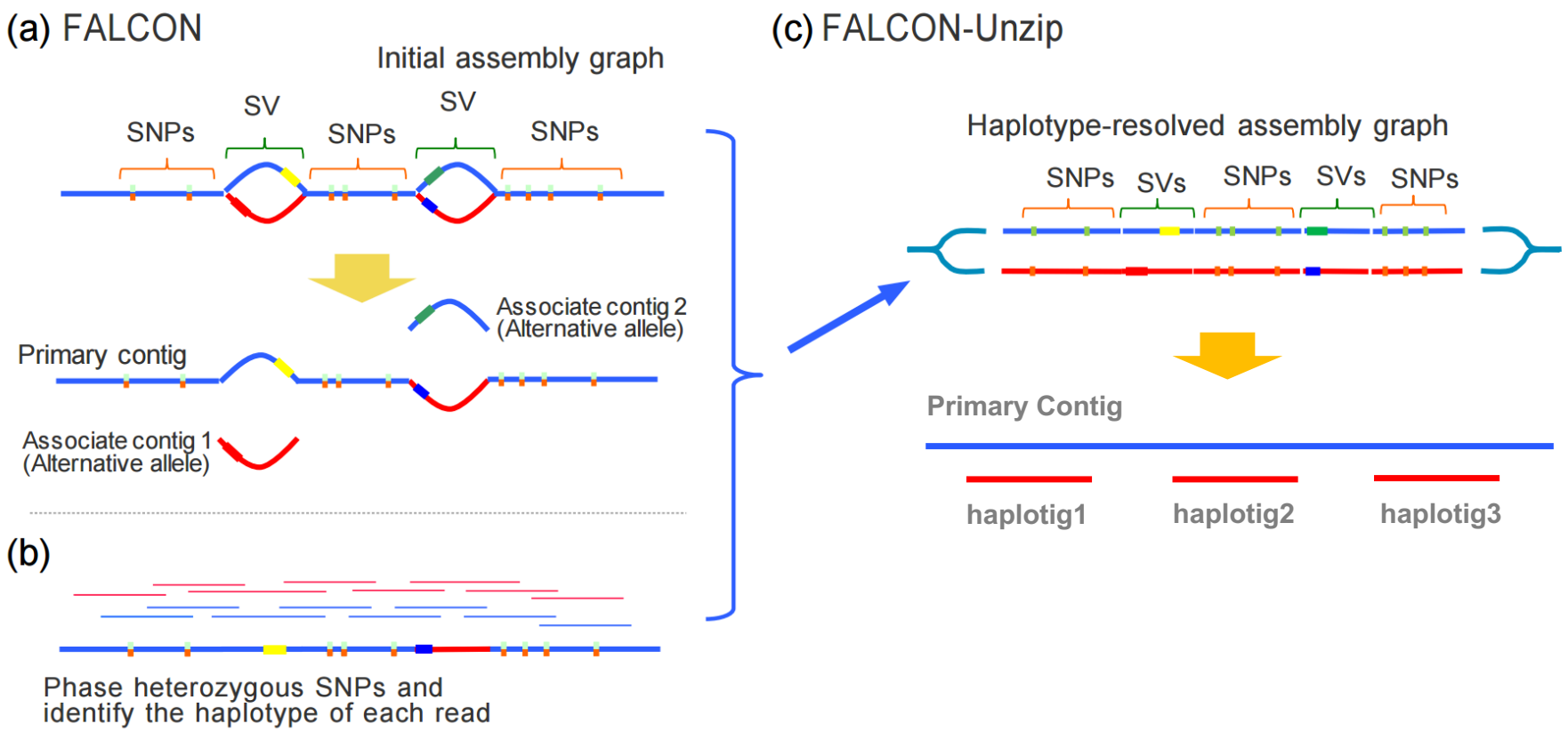
DIPLOID ASSEMBLY WITH FALCON-UNZIP



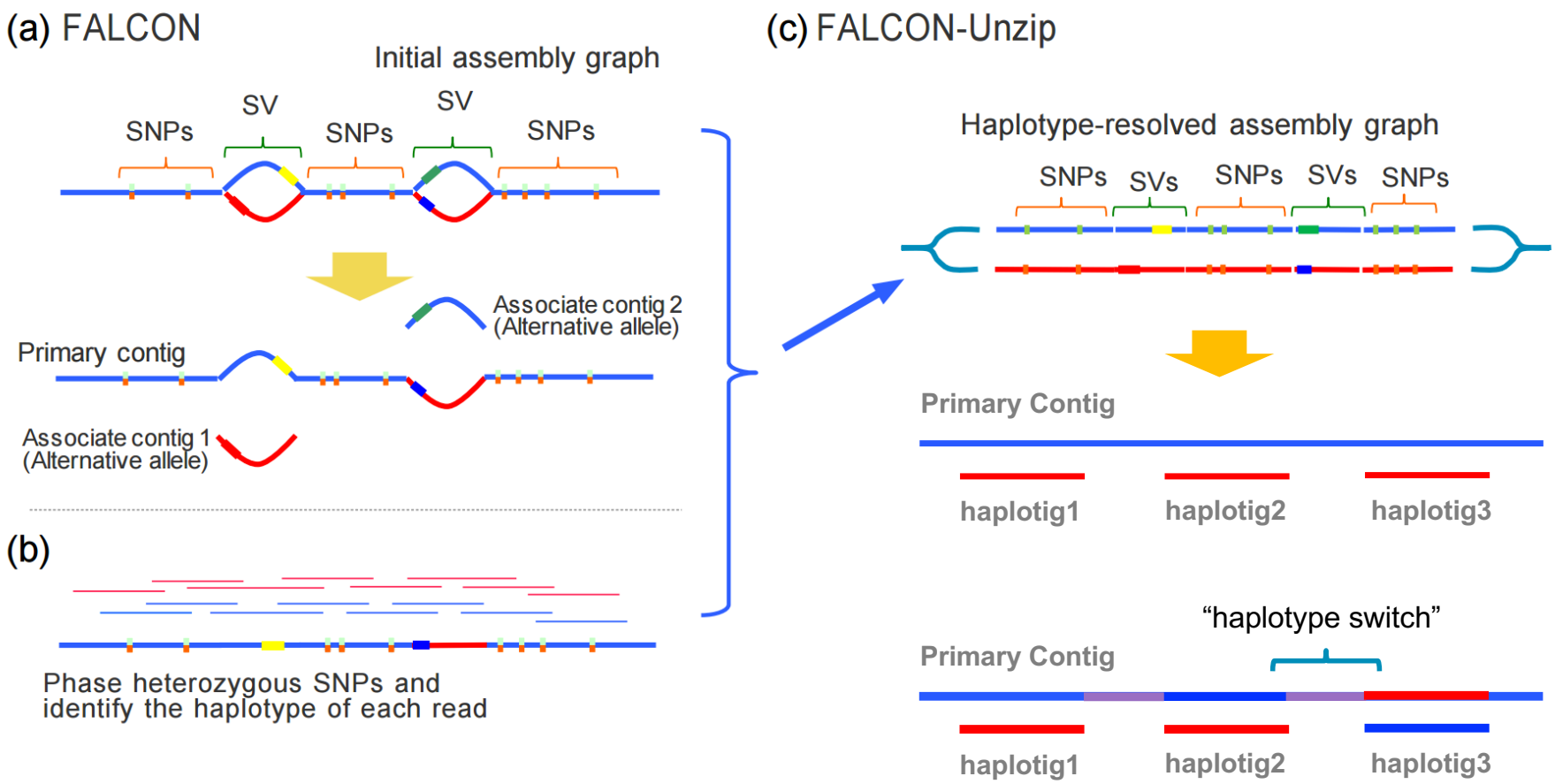
Chin *et al.* (2016). Phased diploid genome assembly with single-molecule real-time sequencing. Nature Methods. 13(12), 1050.

DIPLOID ASSEMBLY WITH FALCON-UNZIP



Chin *et al.* (2016). Phased diploid genome assembly with single-molecule real-time sequencing. Nature Methods. 13(12), 1050.

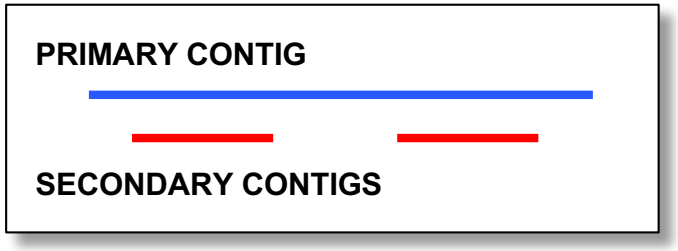
DIPLOID ASSEMBLY WITH FALCON-UNZIP



Chin *et al.* (2016). Phased diploid genome assembly with single-molecule real-time sequencing. Nature Methods. 13(12), 1050.

EXAMPLE ASSEMBLY OF WATER BUFFALO

	FALCON	FALCON-Unzip
Primary Length	2.66 Gb	2.65 Gb
Primary N50	18.7 Mb	18.8 Mb
Secondary Length	0.218 Gb	1.53 Gb
Proportion Phased	8.2 %	58 %



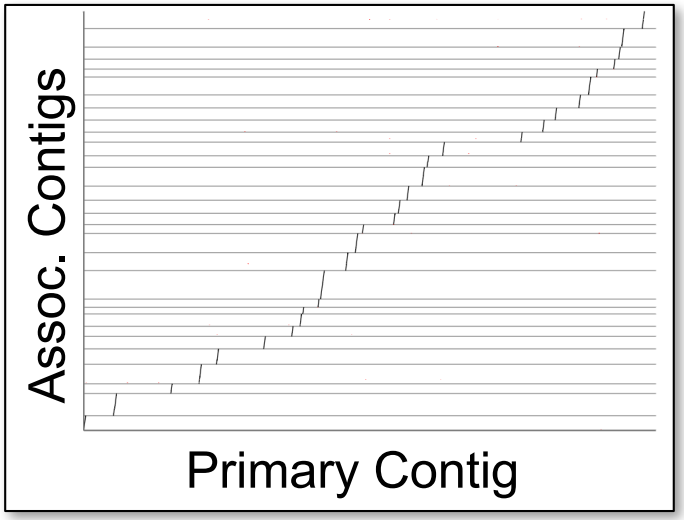
7-fold increase in haplotype phasing with Unzip module

Olimpia Phot Credit: Caterina Cambuli

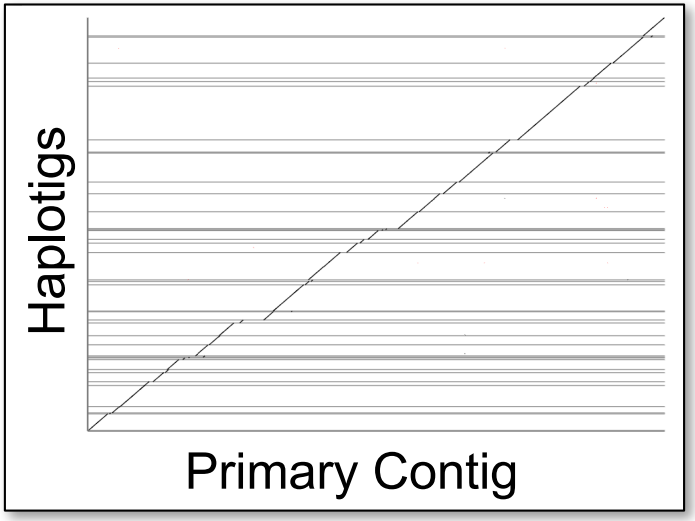
Acknowledgements:
Tim Smith, USDA-ARS
John Williams, Lloyd Low, University of Adelaide
Paolo Ajmone-Marsan, Università Cattolica del S. Cuore
David Hume, Mick Watson, Roslin Institute

INCREASED HAPLOTIG CONTIGUITY WITH FALCON-UNZIP

FALCON



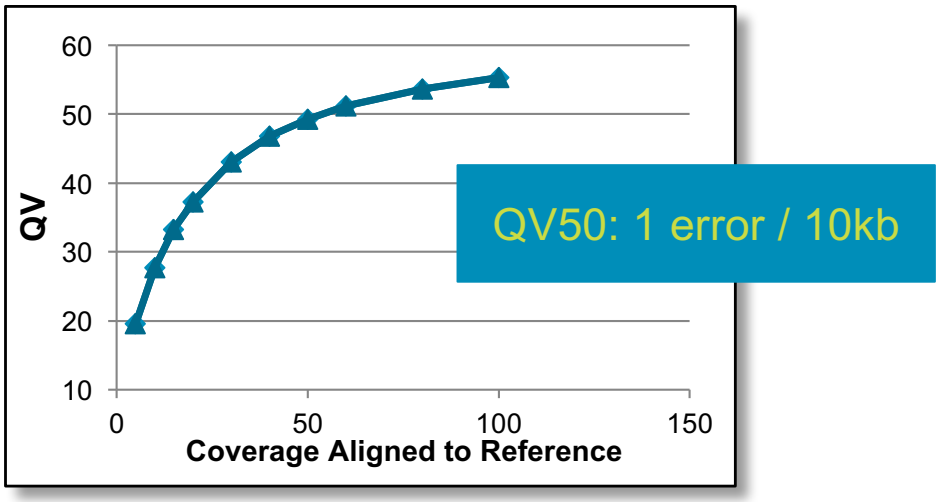
FALCON-UNZIP



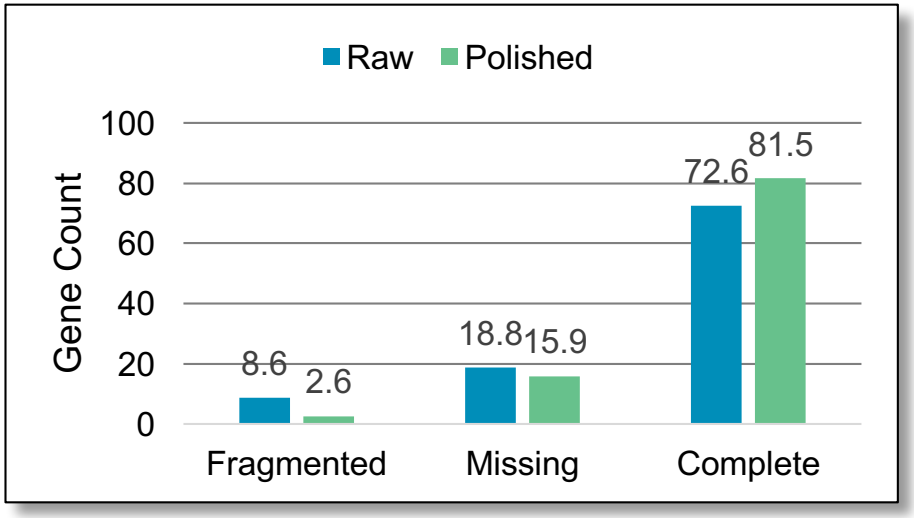
CONTIG: 000078F	FALCON	FALCON-Unzip
Primary Contig Length	12.9 Mb	12.9 Mb
Number Secondary Contigs	30	34
Total Secondary Length	1.21 Mb	10.6 Mb
Secondary Contig N50	42.5 kb	470 kb
Proportion Phased	9.3 %	82%

POLISHING WITH ARROW: INCREASED REFERENCE QUALITY

CONSENSUS BASE ACCURACY



GENOME COMPLETENESS WITH BUSCO



Consensus Base Accuracy

- Sequel 2.0 Chemistry
- Bacterial Genomes

Genome Completeness

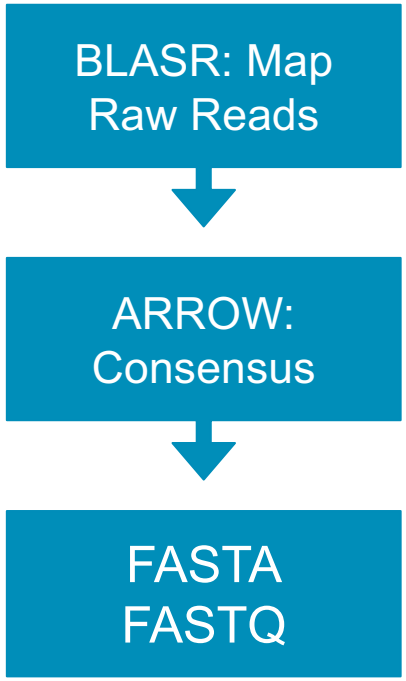
- Avian Genome
- 50-fold Raw Coverage
- BUSCO2 analysis with eukaryota geneset

70% reduction in Fragmented Genes
15% reduction in Missing Genes
12% increase in Complete Genes

Acknowledgement:
Erich Jarvis, Rockefeller University

POLISHING WITH ARROW: WORKFLOW

METHOD	ASSEMBLY	POLISHING
HGAP4 - SMRT Link	√	√
FALCON	√	resequencing pipeline from pbsmrtpipe/SMRT Link
FALCON-Unzip	√	√ (phased) plus optional resequencing



Random Best Mapping

- Random choice of locus with equal BLASR score

Minimum Coverage <5

- <5 reads span 500 bp window
- No consensus call
- Reference base returned as lowercase

Consensus Sequence

atgcgccgttatatgg
aagctagcTAGCTCTA
GTAGCTAGAGCTAGCT
GCGCGCTAGAATAGGG
CGCCATAGAGCCTTTT

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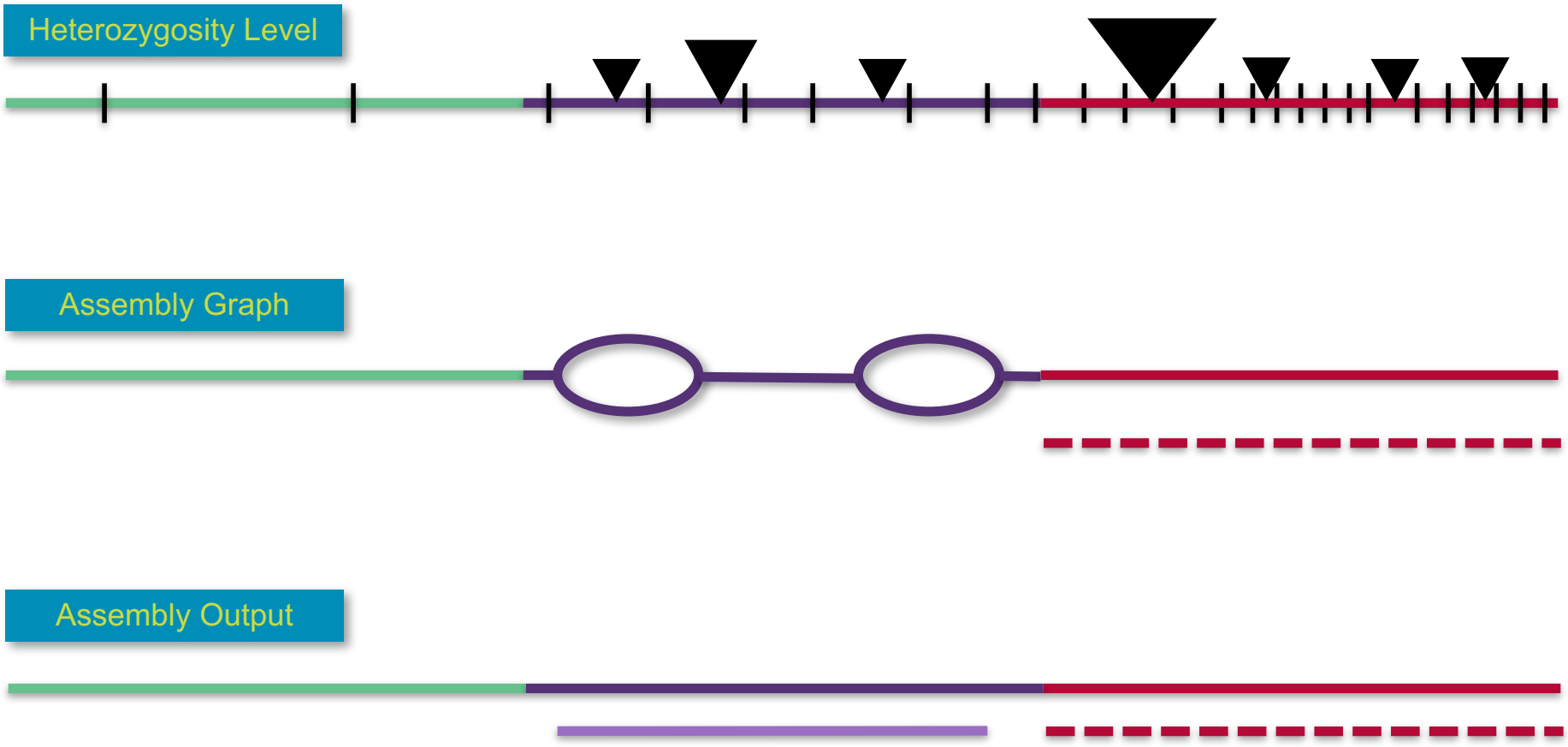
Understanding Your Diploid Assembly

Heterozygosity, Assembly Structure, and Coverage

IMPACT OF HETEROZYGOSITY ON ASSEMBLY PROCESS



Modified from Chin *et al.* 2016

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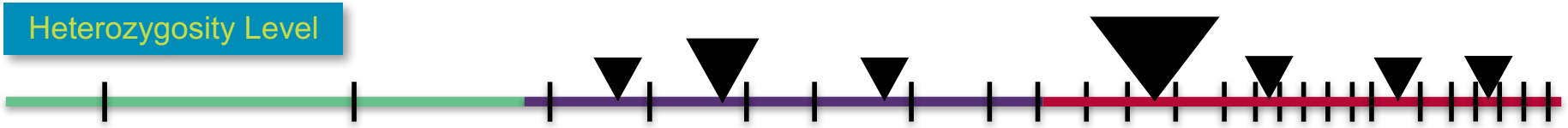

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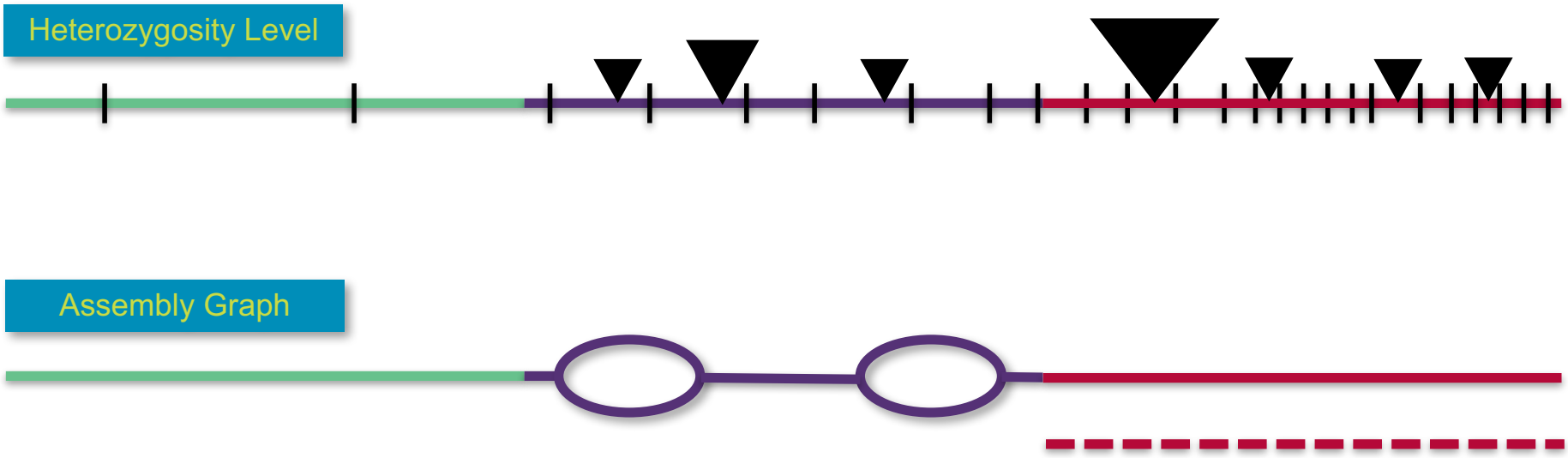

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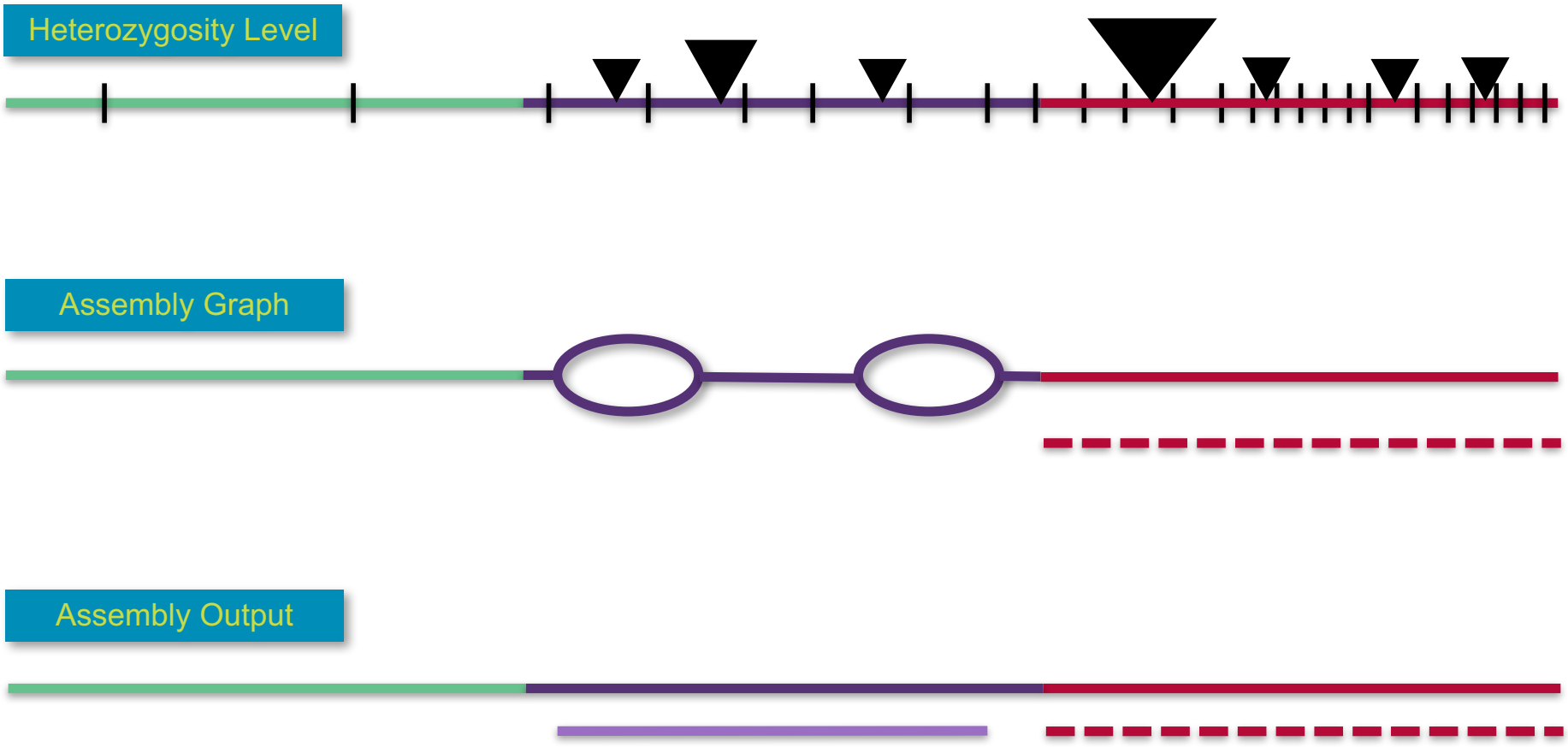
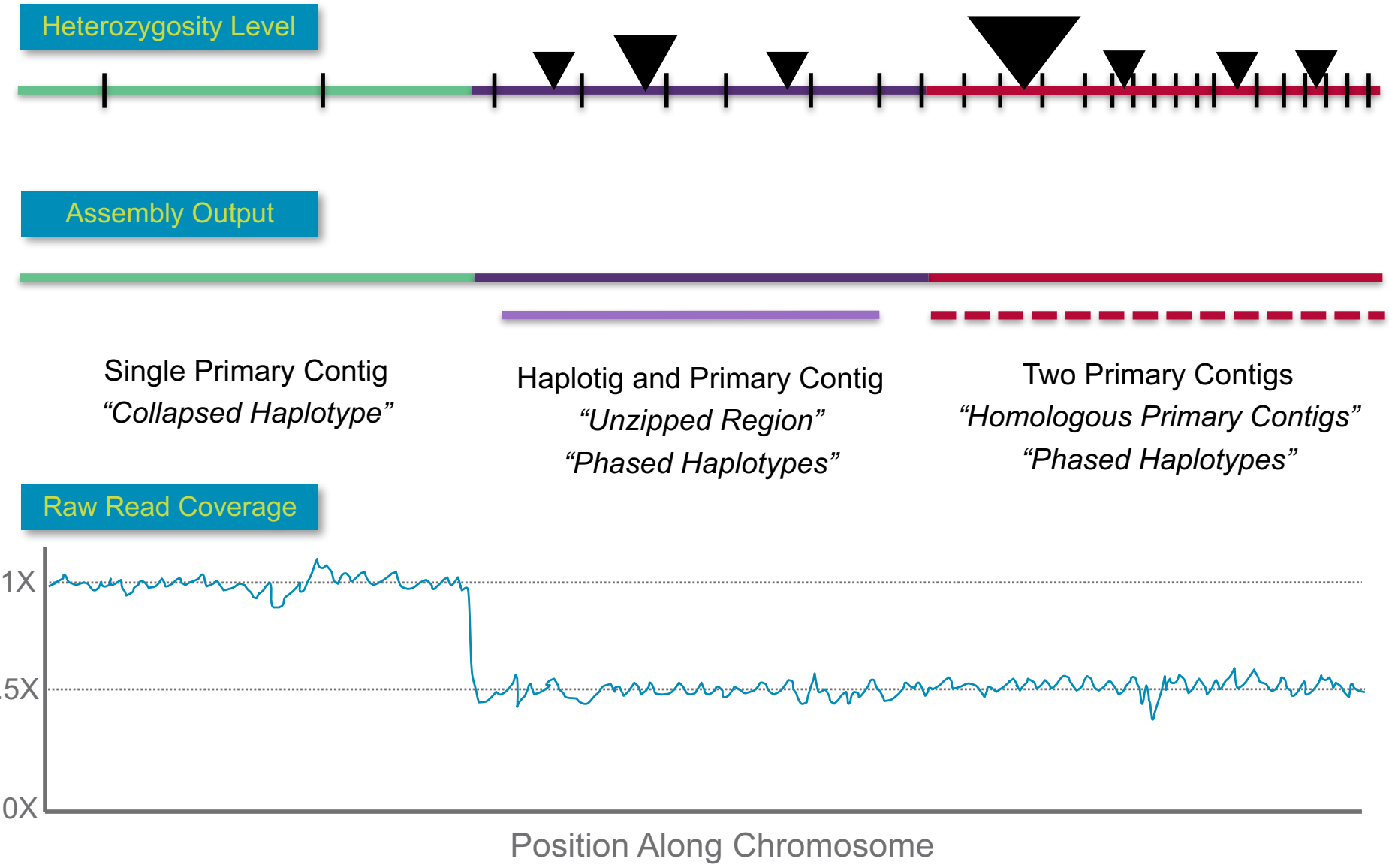


Modified from Chin *et al.* 2016

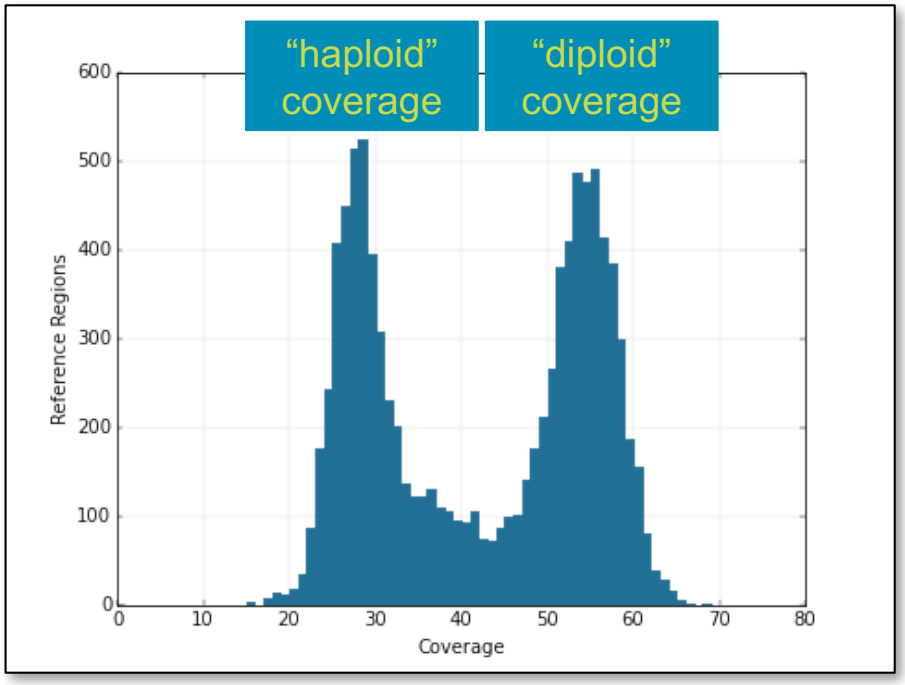
RAW READ COVERAGE AND ASSEMBLY STRUCTURE



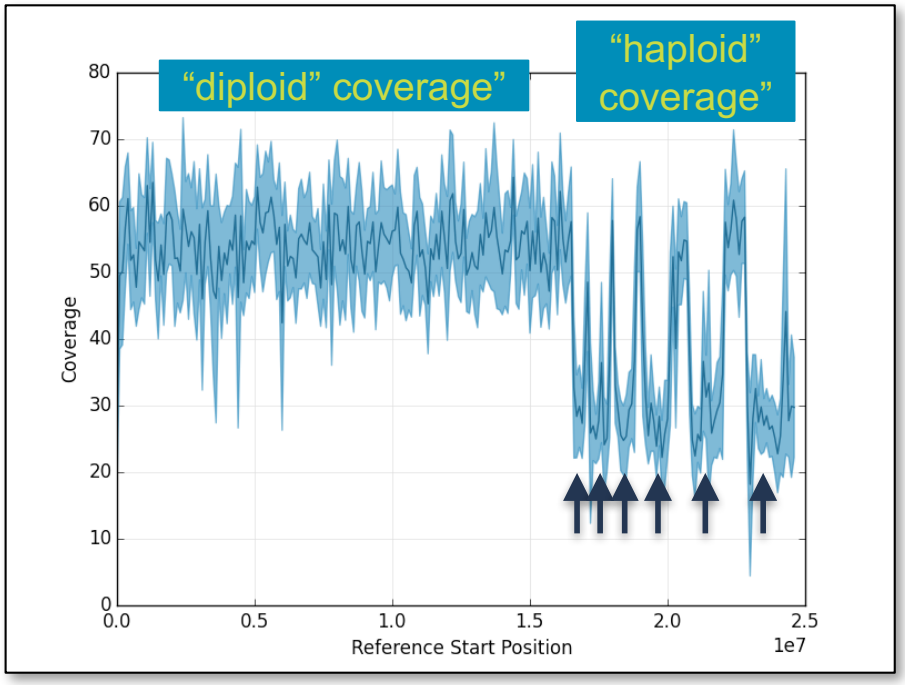
SMRT LINK COVERAGE REPORTS

Graphical Outputs from Resequencing Pipeline / HGAP4

COVERAGE HISTOGRAM: GENOME



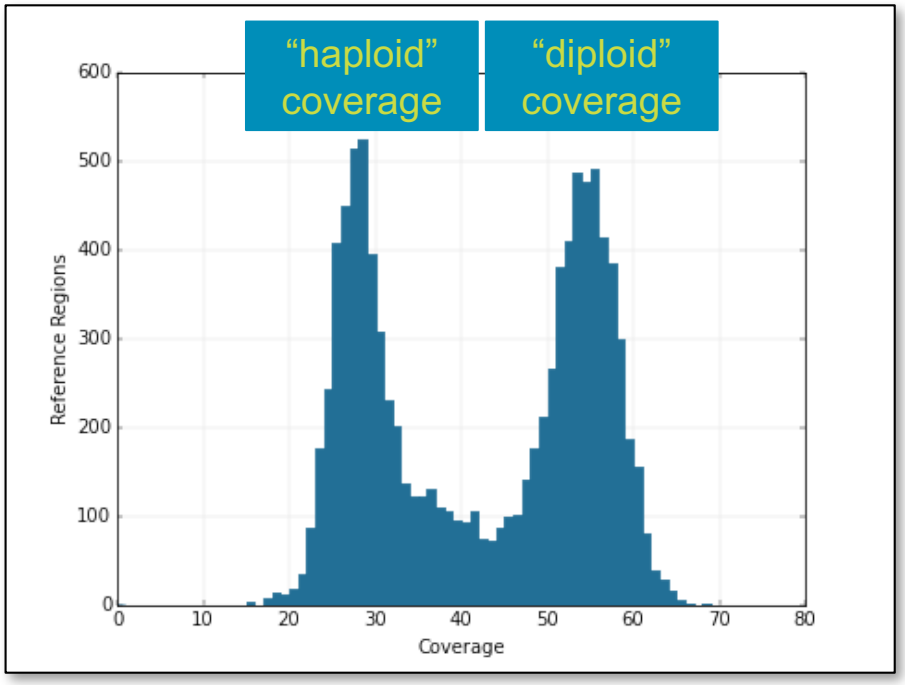
COVERAGE PLOT: CONTIG



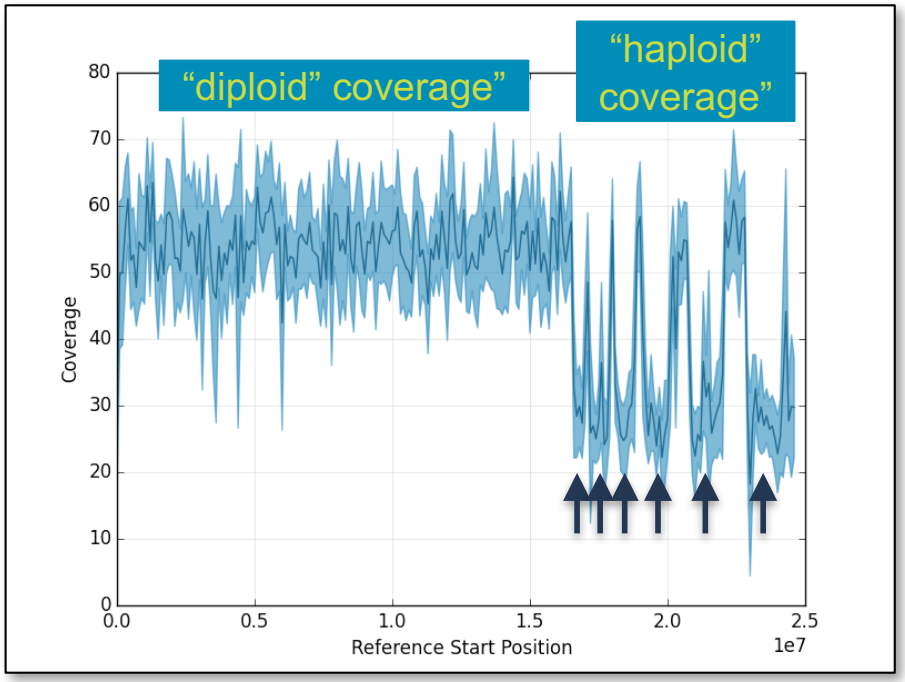
SMRT LINK COVERAGE REPORTS

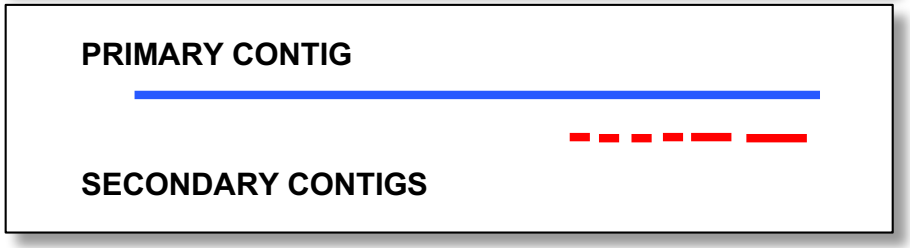
Graphical Outputs from Resequencing Pipeline / HGAP4

COVERAGE HISTOGRAM: GENOME



COVERAGE PLOT: CONTIG

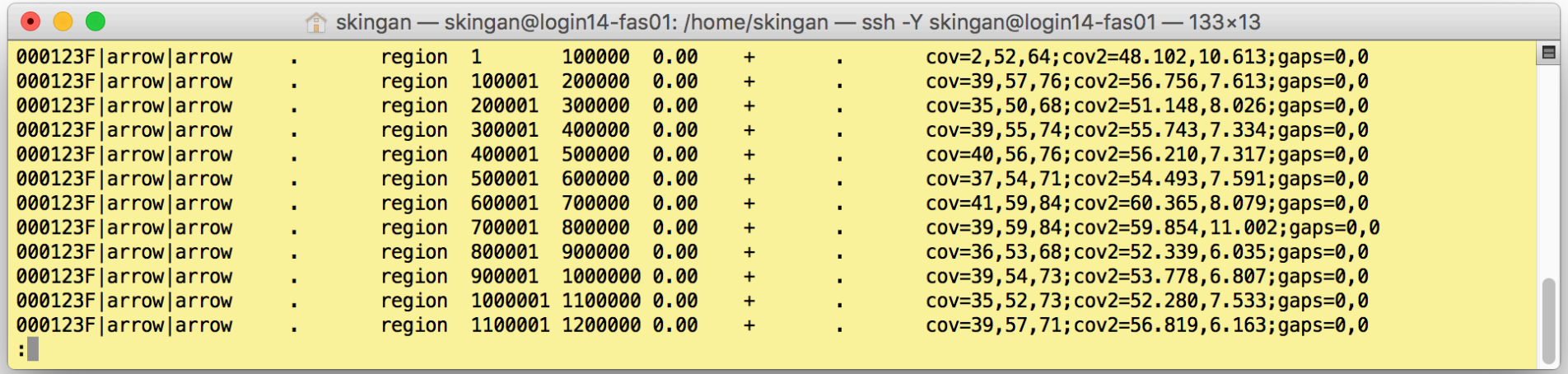




SMRT LINK COVERAGE SUMMARY FILES

alignment_summary.gff: coarse coverage across all contigs

- SMRT Link job directory
  - `myJob/tasks/pbreports.tasks.summarize_coverage-0/alignment_summary.gff`
  - File Format Specs: https://github.com/ben-lerch/SAT

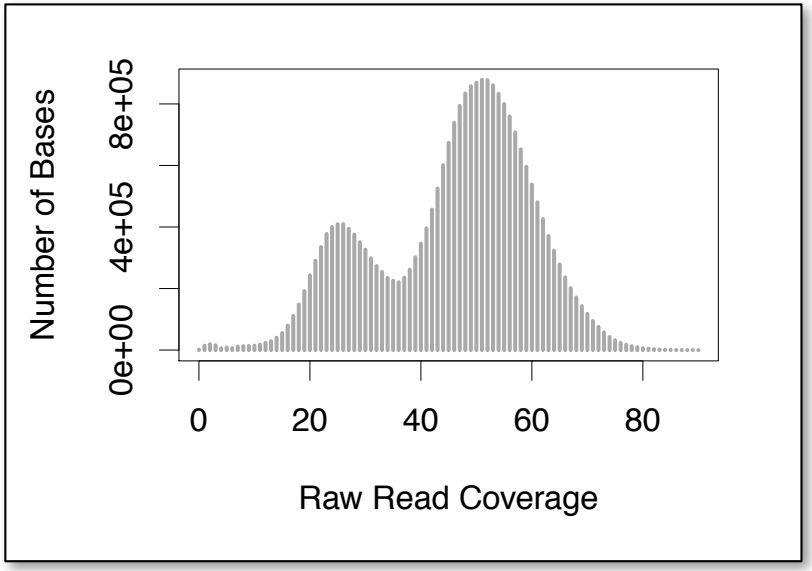


cov	cov2	gaps
min	mean	number continuous gaps
median	s.d.	number gap bases
max		

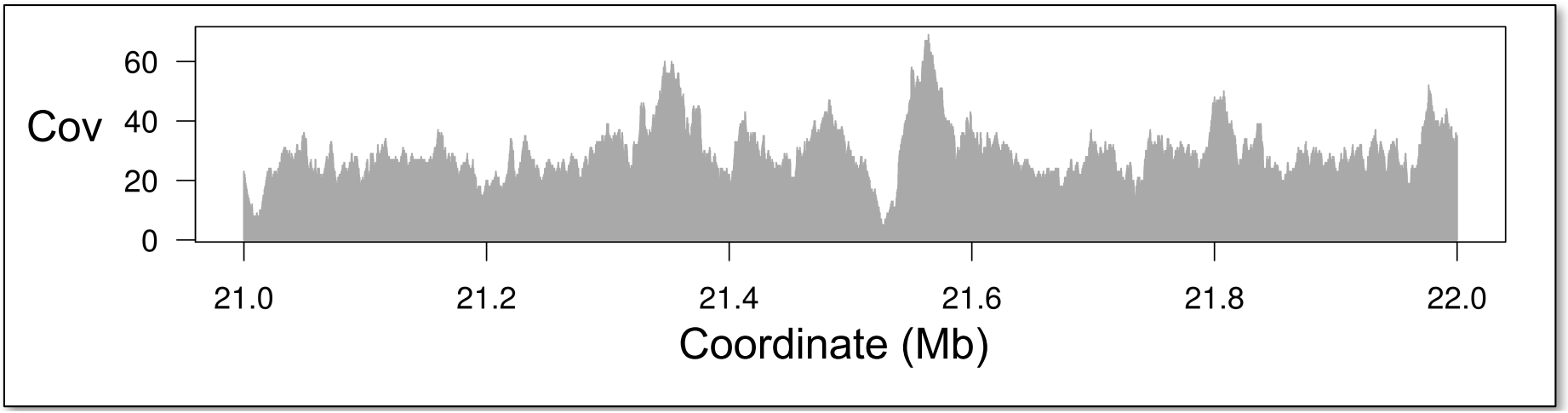
TOOLS FOR CUSTOM COVERAGE ANALYSIS

- Merge BAM files (N=24)
  - `pbmerge`
  - `samtools merge`
- Coverage calculation
  - `samtools depth`
  - `bedtools genomecov`
- Visualization
  - `R – text file/dataframe`

CONTIG COVERAGE HISTOGRAM `bedtools genomecov, R`



CONTIG COVERAGE WINDOW `bedtools genomecov/samtools depth, R`



ALIGNMENTS AND VISUALIZATION

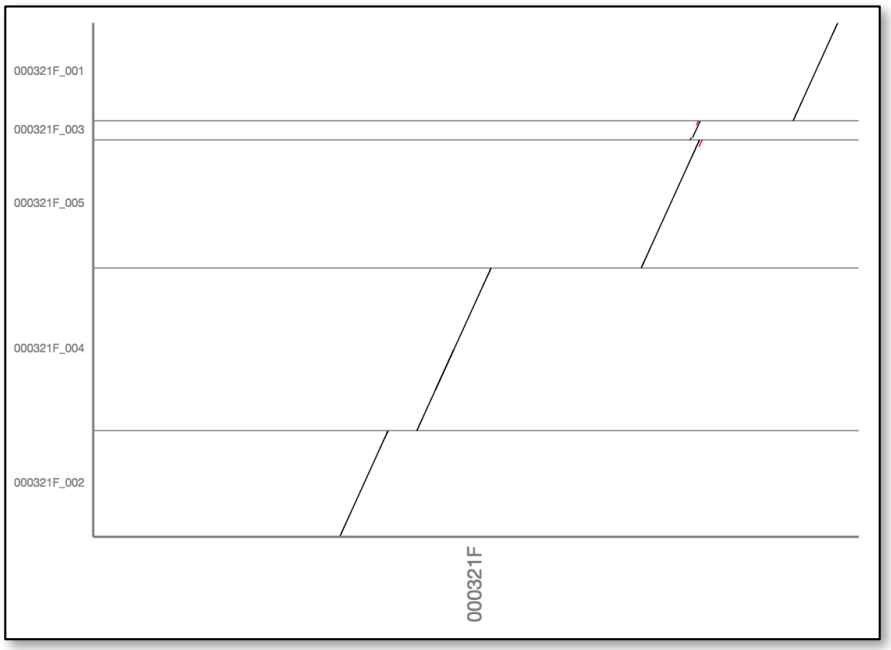
Recommended Tools for Haplotype Alignment and Analysis

- Subset Reference Sequence
  - `samtools faidx`
- Alignment
  - `MUMmer (v4, multi-threaded support)`
  - `NUCmer, delta-filter, show-coords, show-snps, etc`
- Visualization
  - `mummerplot`
  - `assemblytics`
  - `gepard` (alignment + vis)
- FALCON Assembly Tools
  - https://github.com/PacificBiosciences/apps-scripts/
  - FALCONAssemblyTools repo

HAPLOTIGS TO PRIMARY CONTIG DOTPLOT

`alignHaplotigs2Primary.sh`

`Assemblytics`



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Assembly Finishing

Filtering, Circularizing, Haplotype Deduplication

GUIDELINE FOR CONTIG FILTERING

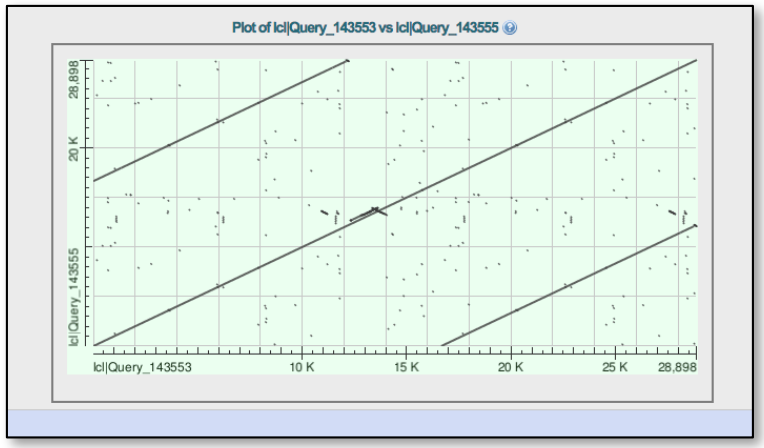
https://github.com/PacificBiosciences/apps-scripts/tree/master/FALCONAssemblyTools

Circularize organelle

- Identify
  - high coverage
  - “circular ctg” FALCON annotation
  - blast hit to organelle
- Circularize and polish
  - minimus2, circulator

SELF-ALIGNMENT OF MITO CONTIG

BLAST



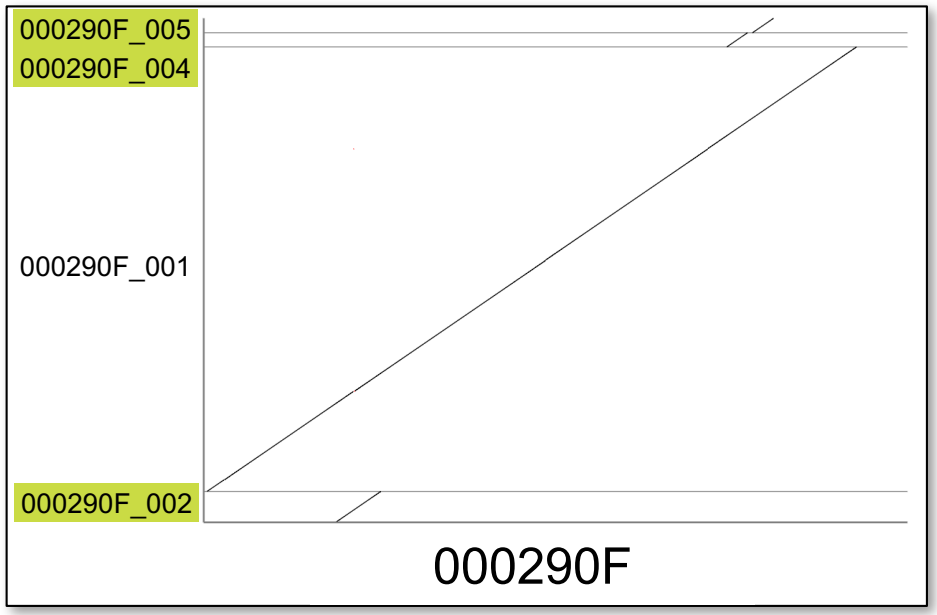
Remove low quality contigs

- Filter out contigs with >50% unpolished bases (lowercase)

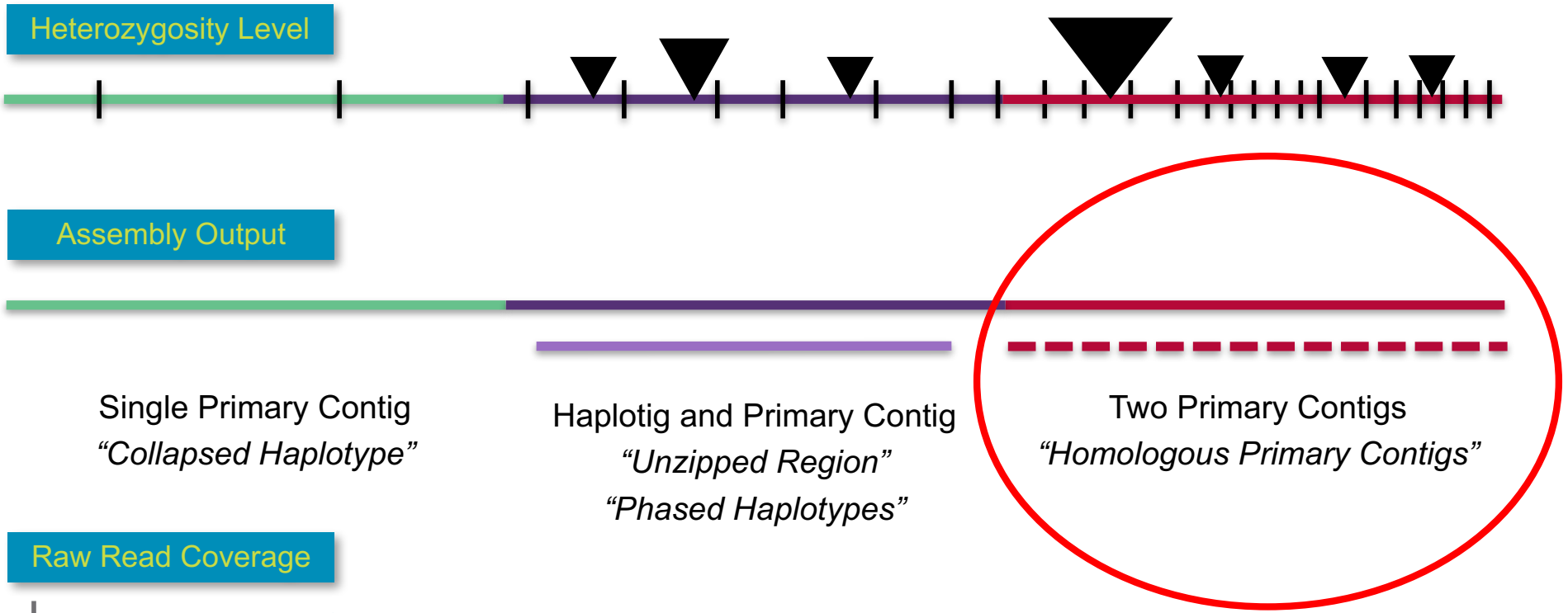
Remove nested haplotigs

- Short haplotigs that align within longer haplotigs

HAPLOTIGS ALIGNED TO PRIMARY



DEDUPLICATING PRIMARY CONTIGS



DOES MY ASSEMBLY HAVE HOMOLOGOUS PRIMARY CONTIGS?

Primary assembly length is longer than haploid genome size

- Inbred individual: diploid assembly: assembly length = 1N
- F1 hybrid: haploid assembly: assembly length = 2N

Haploid coverage on primary contigs in regions *without haplotigs*

BUSCO analysis on primary contigs indicates widespread duplicated genes

METHODS TO IDENTIFY HOMOLOGOUS PRIMARY CONTIGS

BUSCO/Gene Annotation

- Pros: simple, works for highly divergent haplotypes
- Cons: unannotated contigs excluded
- Usage Case: high contiguity assembly, highly divergent haplotypes

All-By-All Alignments

- Pros: simple
- Cons: high compute time/manual curation
- Usage Case: small genome (<1 Gb)

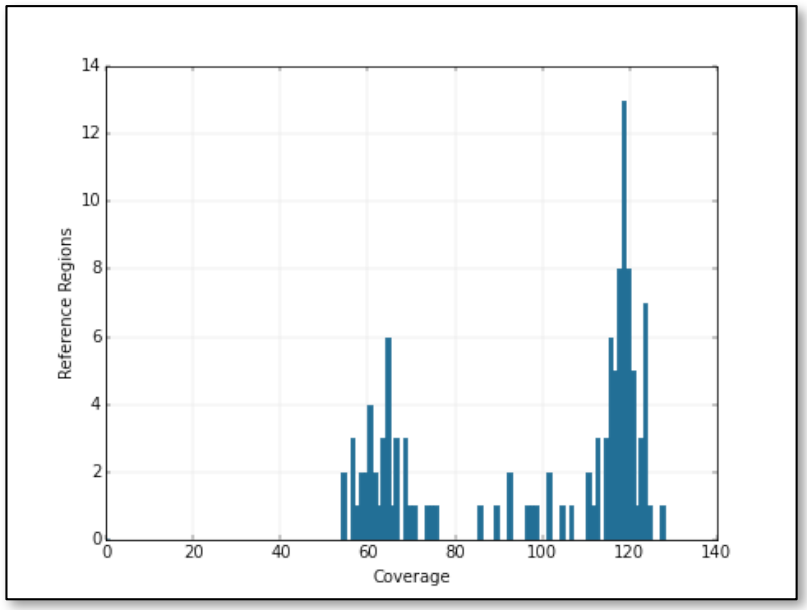
Purge Haplotypes Pipeline (Mike Roach)

- Pros: uses coverage and pairwise identity
- Cons: some manual curation
- Usage Case: many

EXAMPLE: AEDES MOSQUITO FALCON-UNZIP ASSEMBLY

- Expected Genome Size: ~1.3 Gb
- Primary Contig Length: 1.69 Gb

BIMODAL COVERAGE HISTOGRAM



BUSCO ANALYSIS:

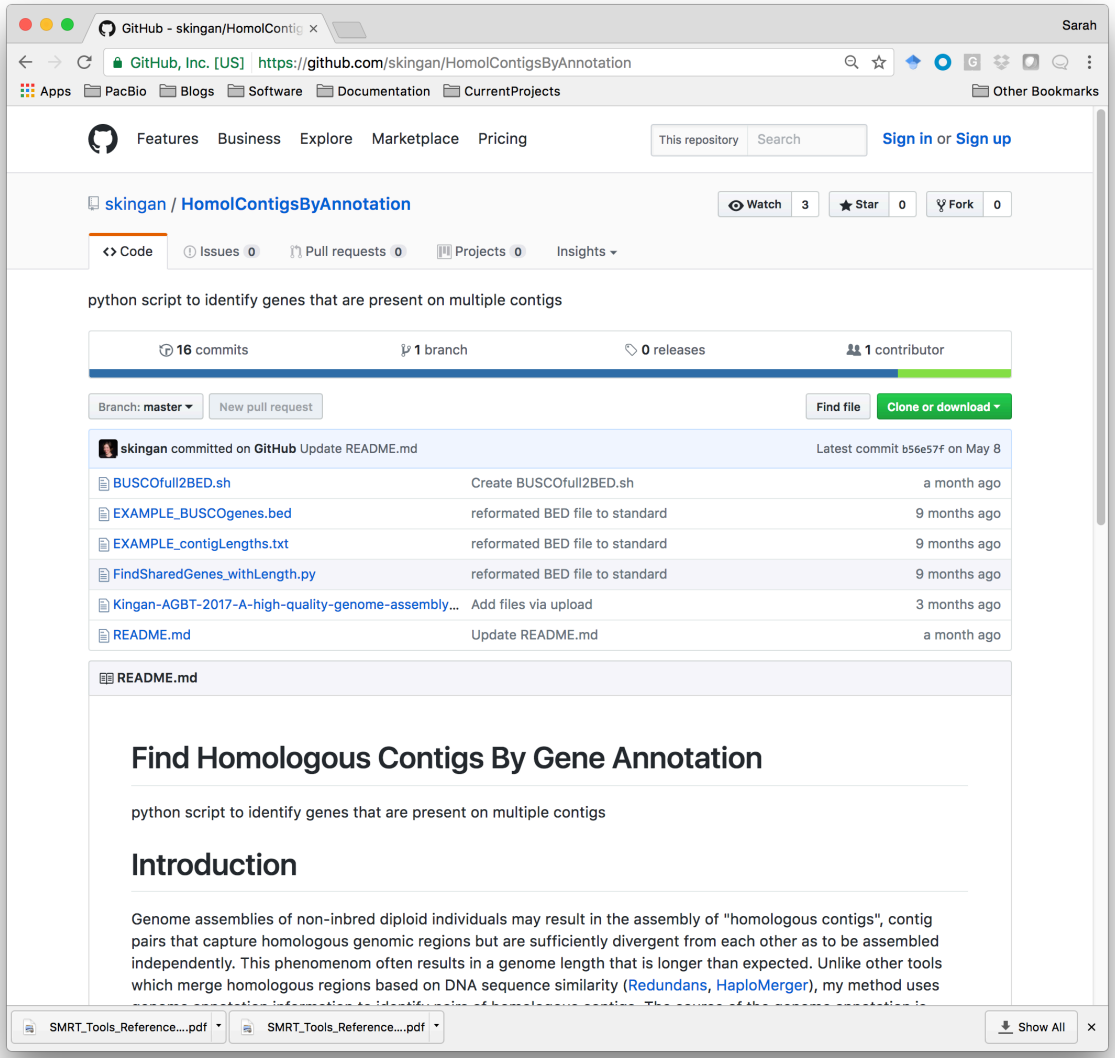
ARTHROPOD GENESET (N = 2675)

ASSEMBLY	*Aedes* PACBIO
COMPLETE	98%
MISSING	2%
FRAGMENTED	10%
DUPLICATED	32%

Acknowledgement:
***Aedes* Genome Working Group**
Leslie Vosshall, Ben Matthews,
Rockefeller University

BUSCO METHOD

github.com/skingan/HomolContigsByAnnotation



Convert BUSCO Output to BED

Input: BED-formatted Gene Annotation

Output: list of contig pairs that share genes

User Curation of Contig Pairs

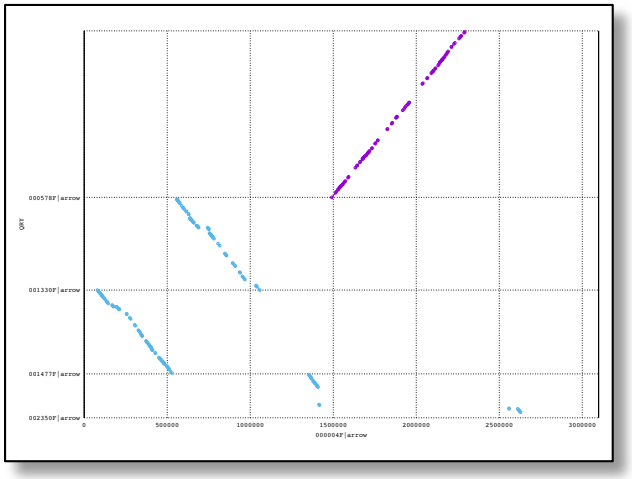
ALL-BY-ALL ALIGNMENTS

https://github.com/PacificBiosciences/apps-scripts/blob/master/FALCONAssemblyTools/get_homologs.py

Identify homologous relationships by alignments

- Each contig aligned to all shorter contigs using NUCmer
  - uses multi-threaded version of MUMmer4
- High quality alignments filtered
- Multi-sequence alignment visualized in mummerplot
- Manual curation of plots

OUTPUT EXAMPLE



PURGE HAPLOTIGS
MIKE ROACH, AUSTRALIAN WINE RESEARCH INSTITUTE

https://bitbucket.org/mroachawri/purge_haplotigs/

Semi-automated pipeline to *remove* haplotigs from primary contigs

- Input: BAM of mapped PacBio reads to primary contigs
- Output: curated **haploid representation** of assembly
  - Record of association between excluded and retained primary contigs

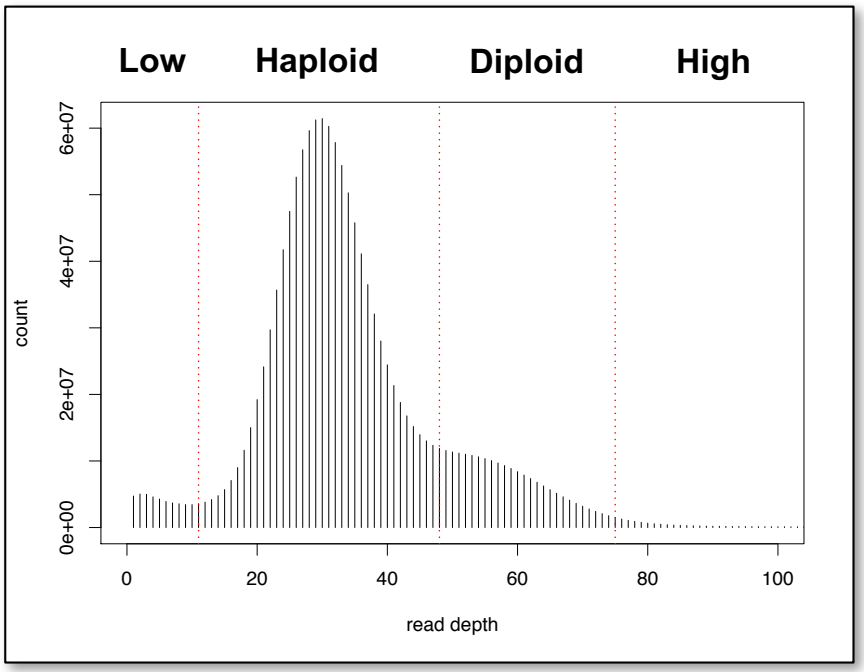
Pipeline

1. coverage histogram and user-defined coverage cut offs
2. contig-specific coverage analysis to identify candidate haplotigs
3. alignments and iterative purging of candidates

PURGE HAPLOTIGS: EXAMPLE FROM BARBERRY BUSH

Acknowledgement:
Iago Hale, UNH

1. PRIMARY CONTIG COVERAGE HISTOGRAM



2. INDIVIDUAL CONTIG COVERAGE ASSESSMENT

- <80% contig length has diploid coverage
- 4470 / 4672 contigs flagged as “suspect”

3. ITERATIVE REASSIGNMENT

- All-by-all BLAST to find two best hits of ”suspect” contigs
- NUCmer alignment and summary stats
- Categorization as “repeat” or “haplotig”

OUTPUT: CURATED ASSEMBLY

- Revised haploid genome
- Log file of reassignment

```
000000F,PRIMARY <- 001282F,REPEAT
                <- 003081F,HAPLOTIG
```

METHODS TO IDENTIFY HOMOLOGOUS PRIMARY CONTIGS

BUSCO/Gene Annotation

- github.com/skingan/HomolContigsByAnnotation

All-By-All Alignments

- https://github.com/PacificBiosciences/apps-scripts/blob/master/FALCONAssemblyTools/get_homologs.py

Purge Haplotypes Pipeline (Mike Roach)

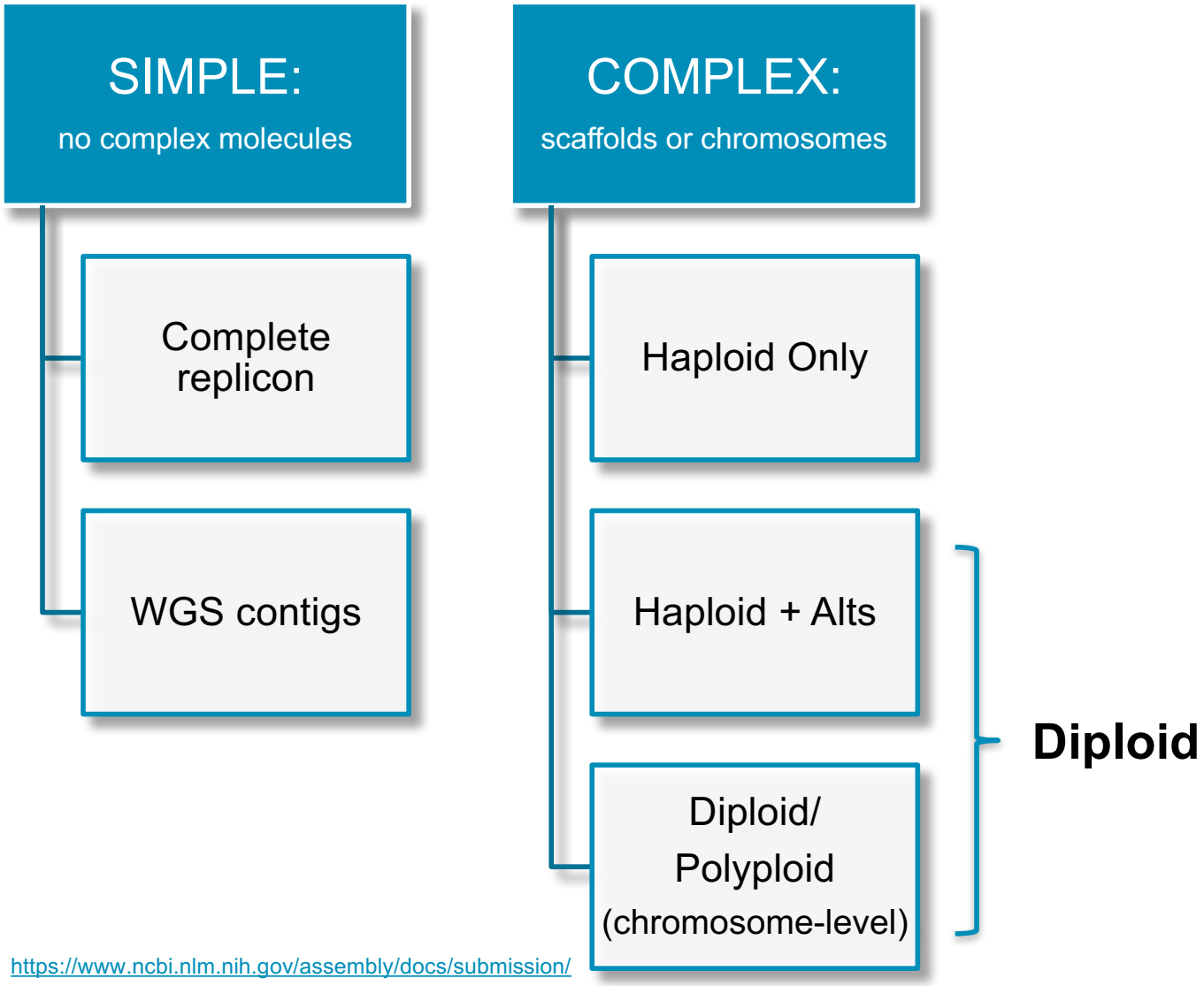
- https://bitbucket.org/mroachawri/purge_haplotigs/

PACBIO®

Sharing Your Assembly

Diploid Assembly Submission to NCBI

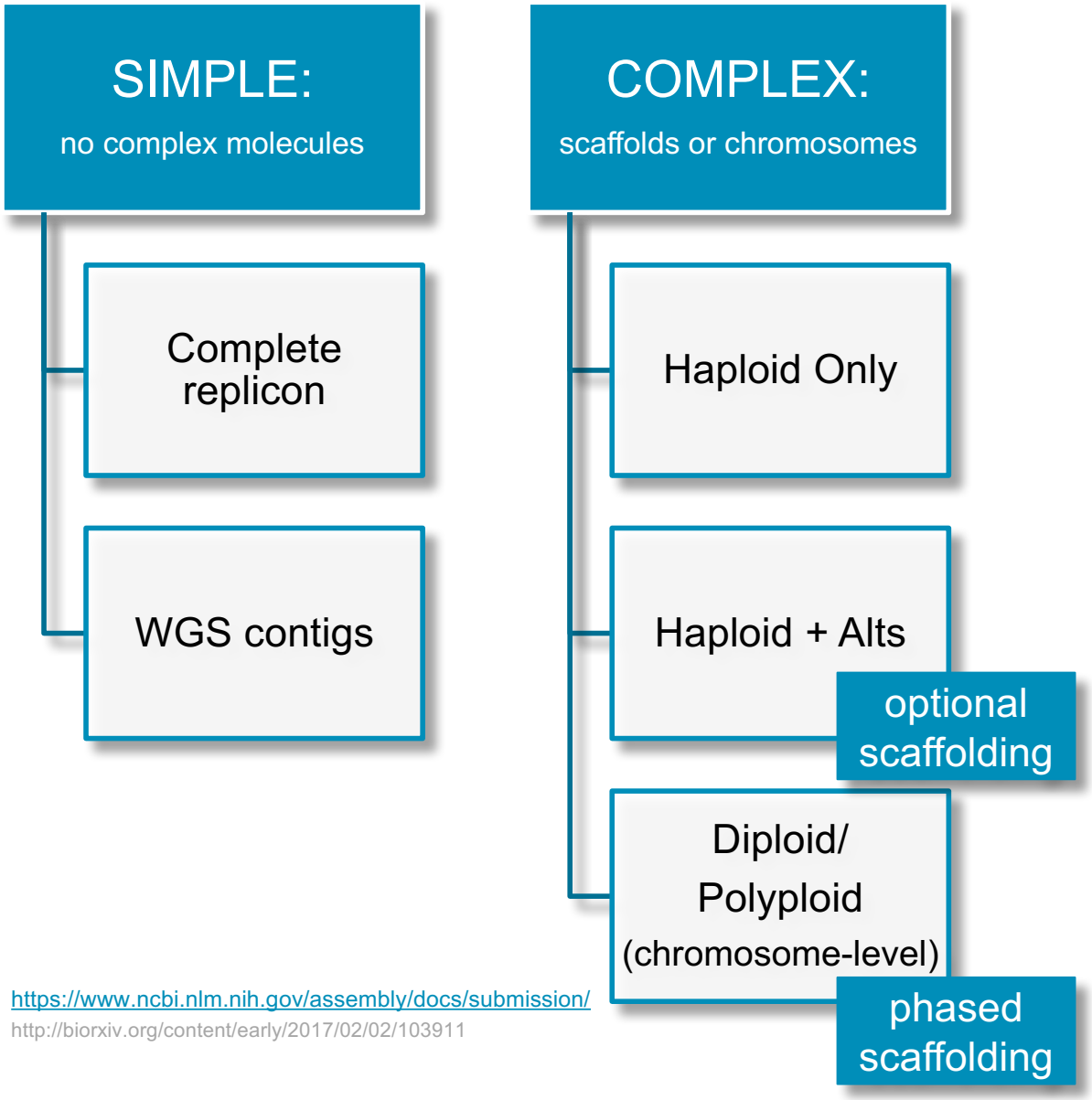
ASSEMBLY SUBMISSION TYPES



https://www.ncbi.nlm.nih.gov/assembly/docs/submission/

http://biorxiv.org/content/early/2017/02/02/103911

ASSEMBLY SUBMISSION TYPES



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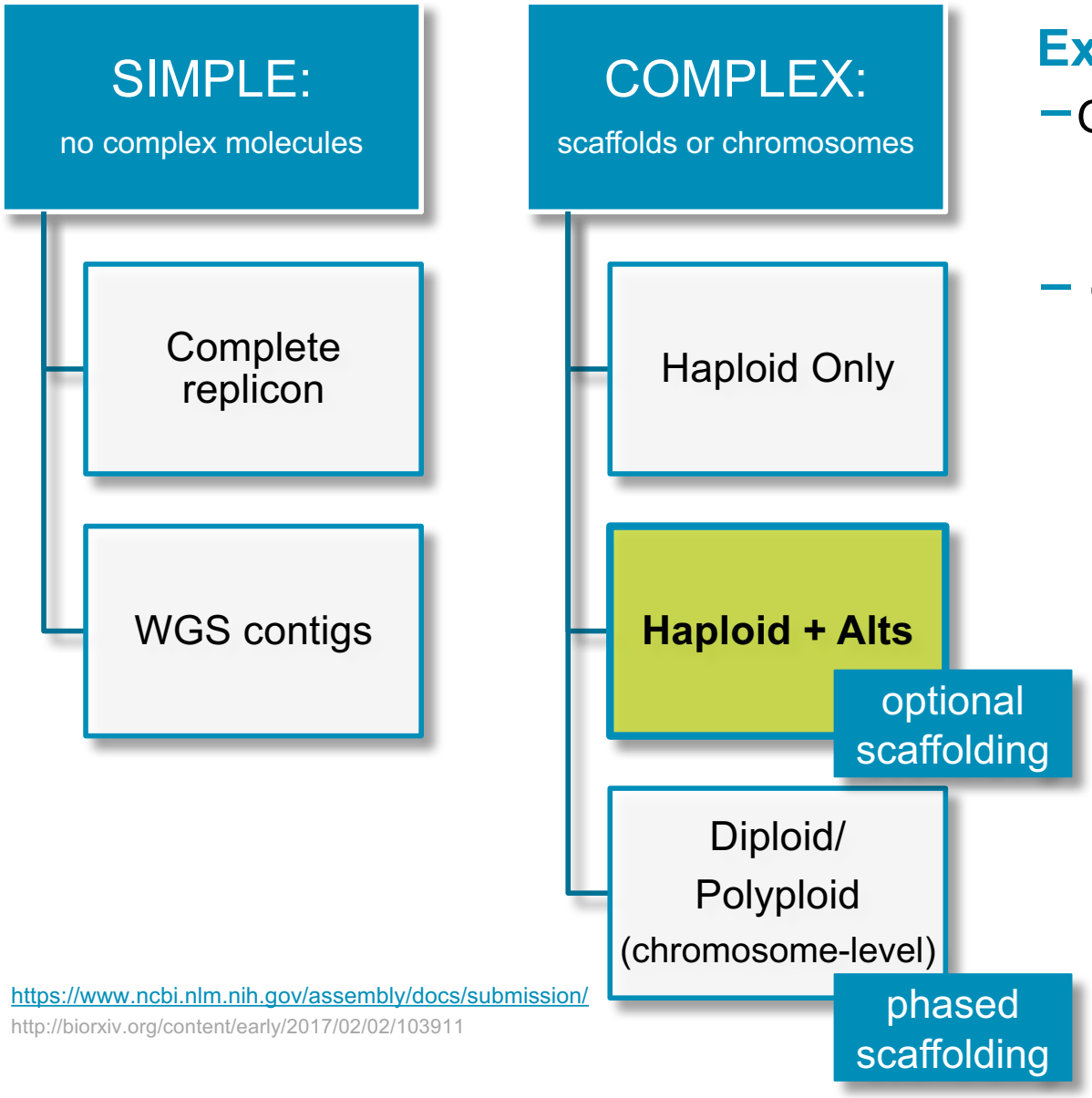
ASSEMBLY SUBMISSION TYPES



https://www.ncbi.nlm.nih.gov/assembly/docs/submission/

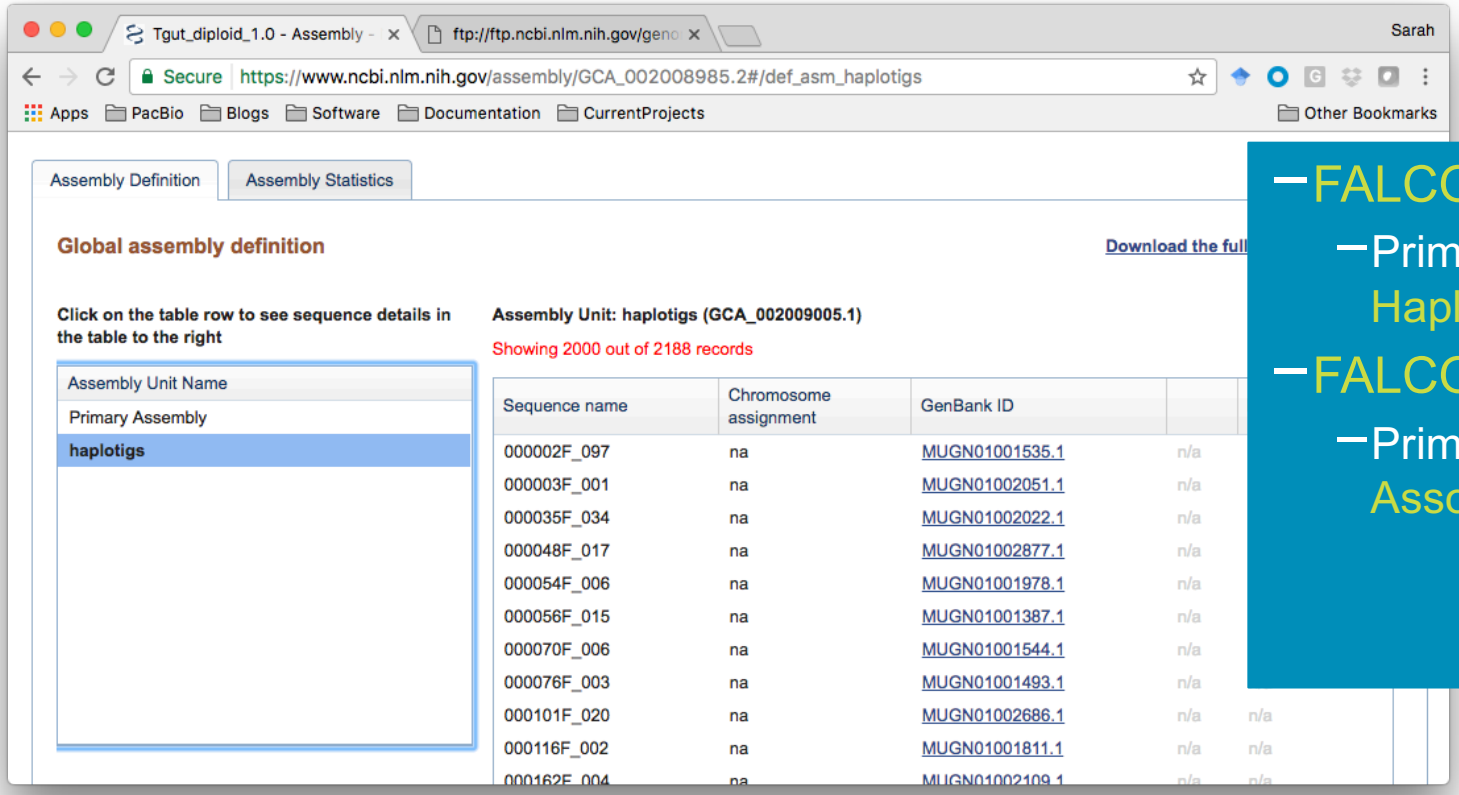
http://biorxiv.org/content/early/2017/02/02/103911

Examples of Haploid + Alts

- GCA_001753755.2
  - *Arabidopsis thaliana* F1 from Chin *et al.* 2016
- GCA_002008985.2
  - zebra finch Korlach *et al.* 2017

ASSEMBLY UNITS: PRIMARY AND ALT

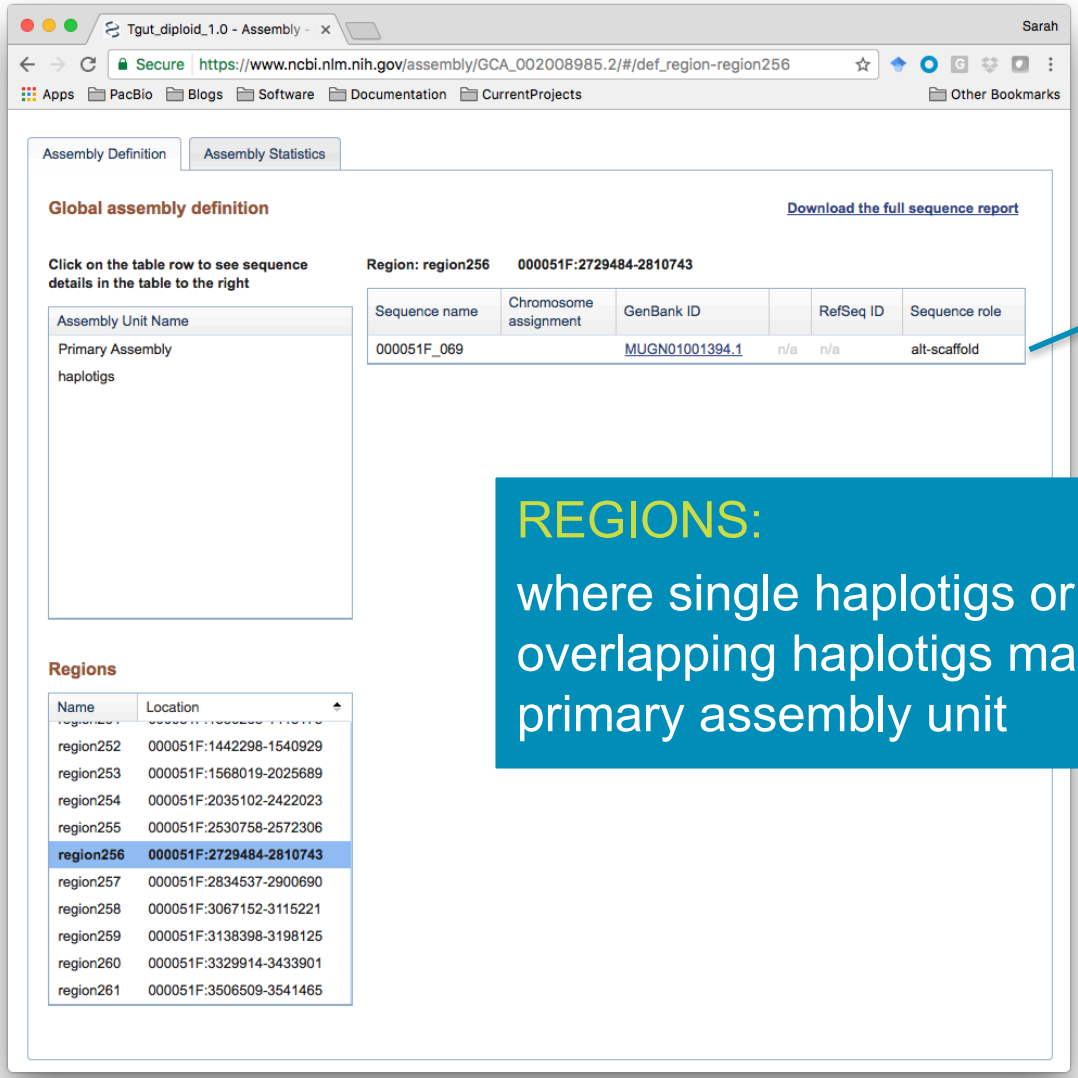
Zebra finch: GCA_002008985.2

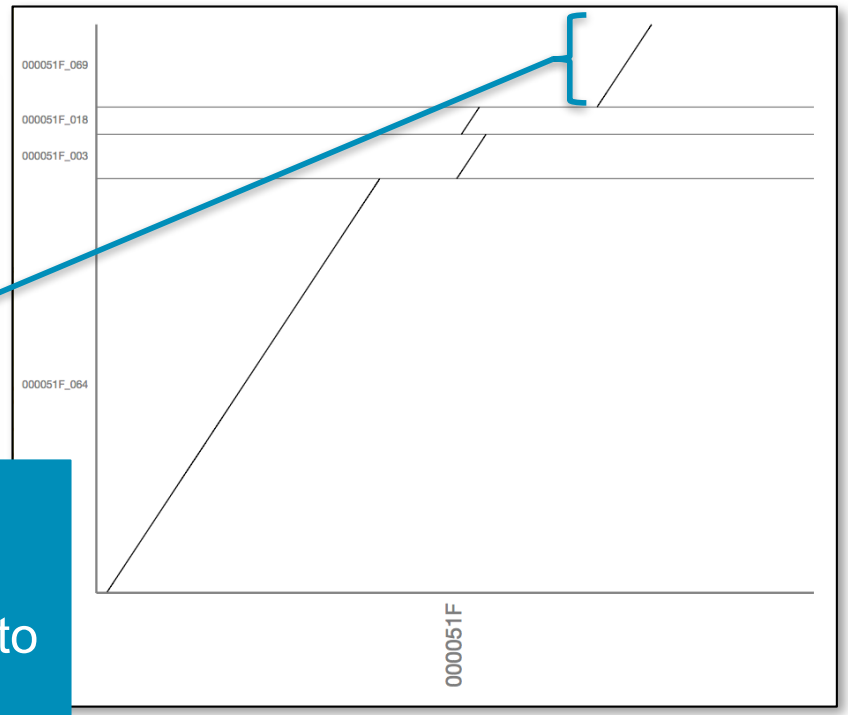


- FALCON-Unzip:
  - Primary Contigs and Haplotigs
- FALCON:
  - Primary Contigs and Associated Contigs

REGIONS

Zebra finch: GCA_002008985.2



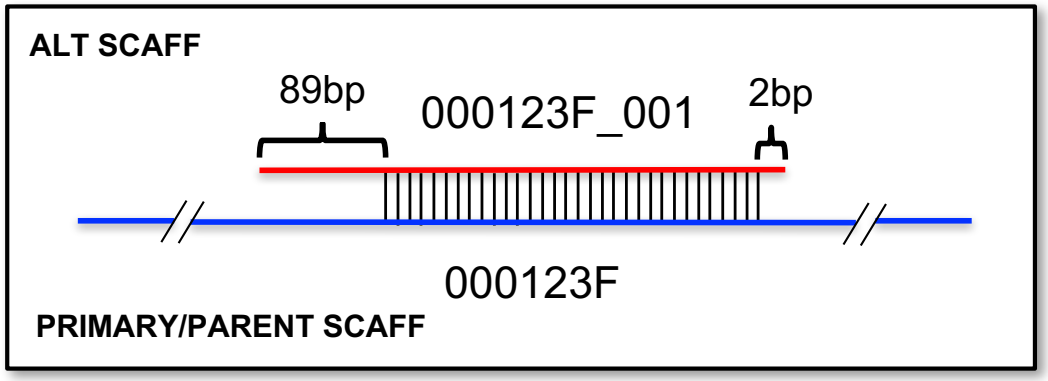


REGIONS:

where single haplotigs or overlapping haplotigs map to primary assembly unit

ALTERNATE LOCUS PLACEMENT FILE

- Required for haploid + alts submission
- Details placement of alt sequences relative to primary assembly



HEADER	EXAMPLE
alt_asm_name	haplotigs
prim_asm_name	Primary Assembly
alt_scaf_name	000123F_001
parent_type	SCAFFOLD
parent_name	000123F
ori	+
alt_scaf_start	90
alt_scaf_stop	41595
parent_start	8663681
parent_stop	8708427
alt_start_tail	89
alt_stop_tail	2

PLACEMENT FILE TOOLS

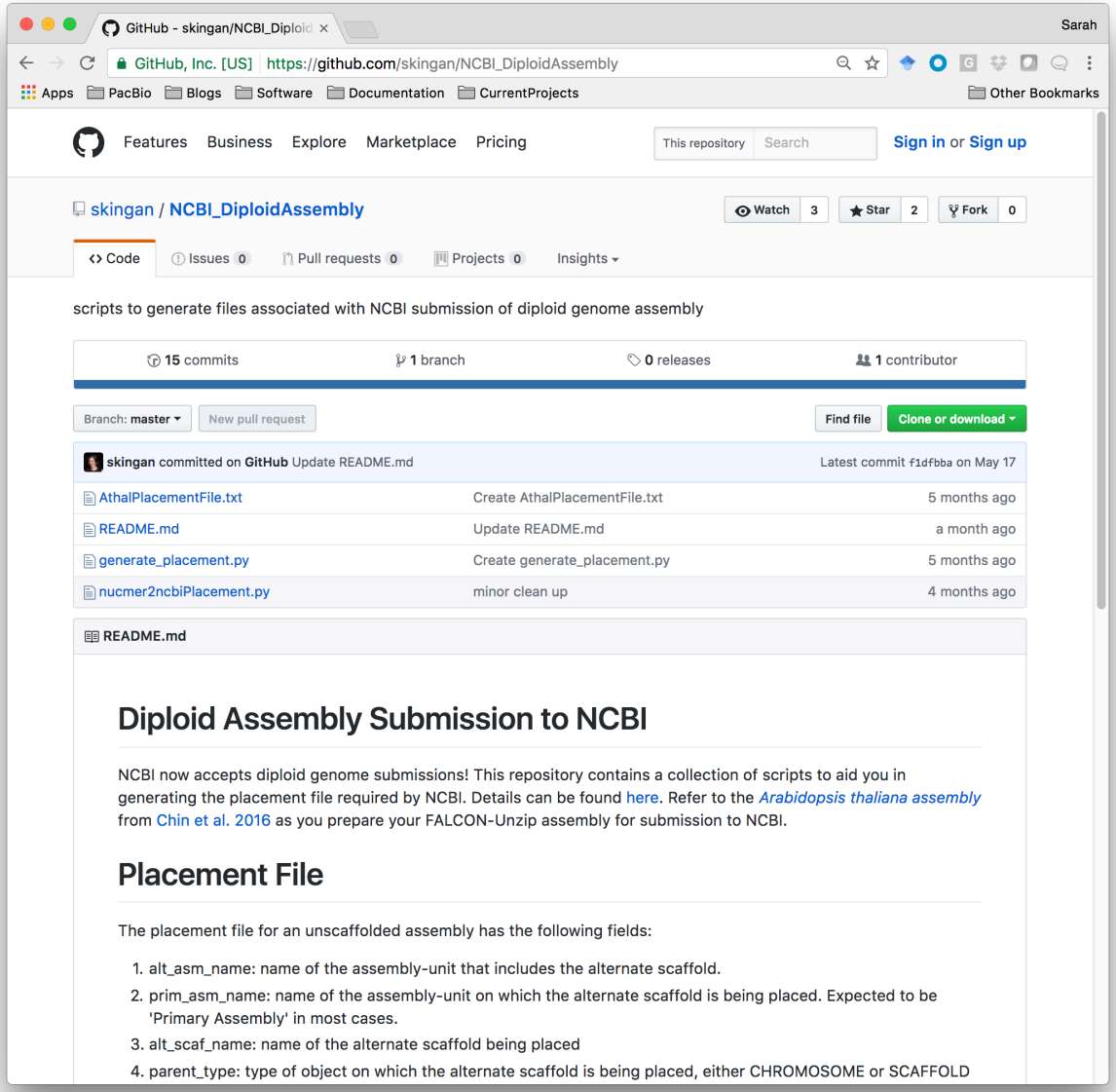
https://github.com/skingan/NCBI_DiploidAssembly

generate_placement.py

- Written by Jason Chin
- Runs NUCmer and generates placement file

nucmer2ncbiPlacement.py

- Written by Sarah Kingan
- generates placement file from directory of filtered NUCmer alignments
- Contains suggested MUMmer commands



RESOURCES

FALCON

- http://pb-falcon.readthedocs.io/
- https://github.com/PacificBiosciences/FALCON-integrate
- https://github.com/PacificBiosciences/apps-scripts/tree/master/FALCONAssemblyTools
- ***Unzip binary to be released with SMRT Analysis 5.0 in Q3***

SMRT Analysis

- http://www.pacb.com/support/software-downloads/
- http://programs.pacificbiosciences.com/l/1652/2017-02-01/3rzxn6/184345/SMRT_Tools_Reference_Guide__v4.0.0_.pdf

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