Assembly Tutorial

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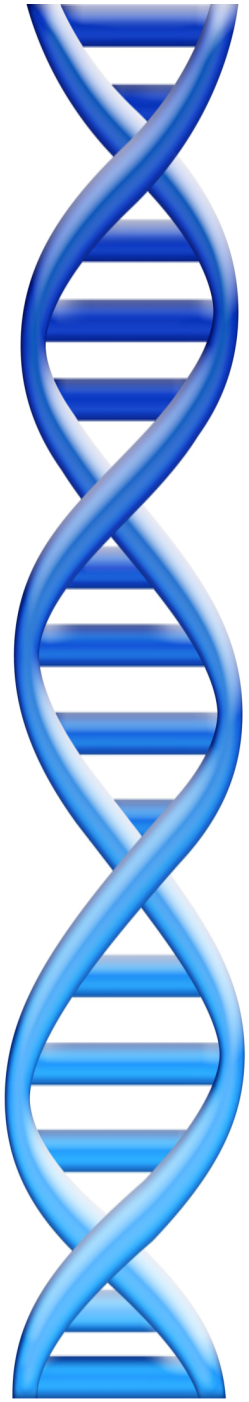
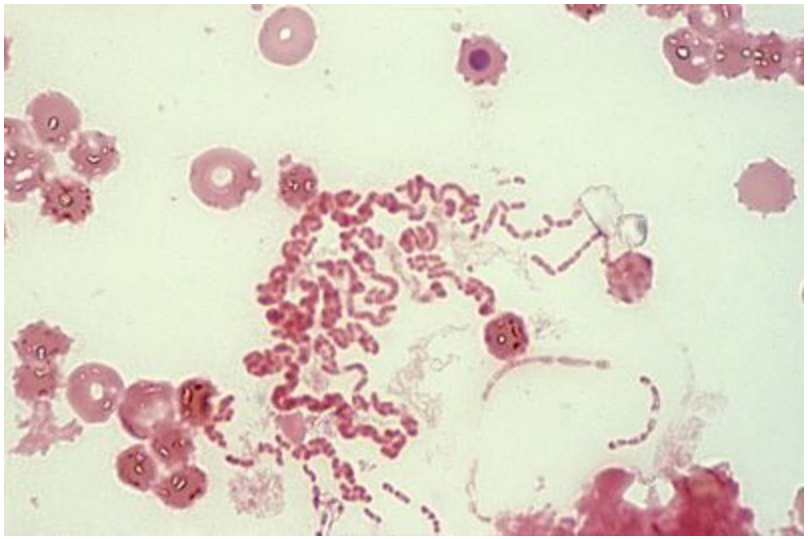
CSHL Sequencing Course



Outline

1. Sample Data
2. ALLPATHS-LG
3. SOAPdenovo + Quake
4. MUMmer

Rhodobacter sphaeroides



Genome architecture:

- Chr1: 3Mbp, Chr2: 900kbp, 5 plasmid
- High GC: 69%

Library 1: Fragment

Avg Read length: 101bp

Insert length: 180bp

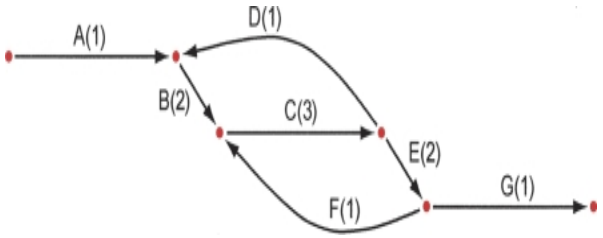
# of reads: 2,050,868

Library 2: Short jump

Avg Read length: 101bp

Insert length: 3500bp

# of reads: 2,050,868



Running ALLPATHS-LG

Iain MacCallum

BROAD INSTITUTE

ALLPATHS-LG mission

Goal

- high quality ‘automated’ genome assembly from low-cost data

How

- define sequencing model to optimize results
- develop algorithm that works well on these data
- change as sequencing technology changes

DNA source

ALLPATHS-LG assumes that the DNA comes from a single individual or culture (one genome)

Mixed populations = mixed genomes

viral patient sample

many individuals from a species all squished together

many species (metagenomic)

These types of data would require a different algorithm....

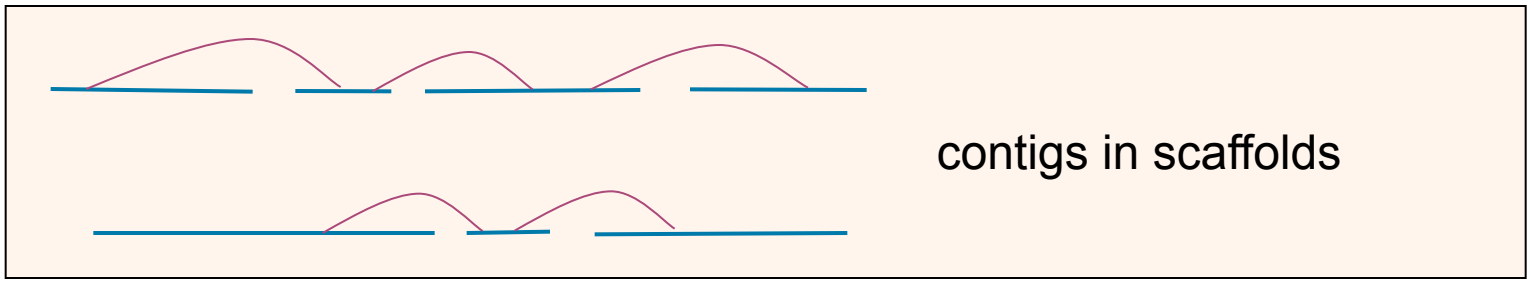
How to use ALLPATHS-LG

1. **What is an ALLPATHS-LG assembly?**
2. Data requirements
3. Computational requirements
4. Installation
5. Preparing your data
6. Assembling

Three models for assembly

1. Linear assemblies
2. Graph assemblies
3. Linearized graph assemblies (ALLPATHS-LG)

1. Linear assemblies



contig: a contiguous sequence of bases….

```
CTGCCCCCTGTGCCAATGGGTTTGAGGCTCTTCCCACTTCCTTTTCTATTAGATTCAATGTATCTGGTTTTATGTTGAGG
TCCTAGATCCACTTGGACTTGAGCTTTGTACAAGATGACATATATAGGTCTGTTTTTATTCTTCTACATACAGACAGCCA
GTTATACCAGCACCATTTATTGAAGACACTTTCTTTATTCCATTGTATATTTTTTTACTTCCTTGTCAAAAATCAAGTGA
CCATGAGTATGTGGTTTCATTTCTGGGTCTTCAATTGTATTCCATTAGTCAACATATCTGTCTCTGTACCAATACCATGC
```

scaffold: a sequence of contigs, separated by gaps….

```
TCCTAGATCCACTTGGACTTGAGCTTTGTACAAGATGACATATATAGGTCTGTTTTTATTCTTCTACATACAGACAGCCA
GTTATACCAGCACCATTTATTGAAGACACTTTCTTTATTCCATTGTATATTTTTTTACTTCCTTGTCAAAAATCAAGTGA
CCATGAGTATGTGGTTTCATTTCTGGGTCTTCAATTGTATTCCATTAGTCAACATATCTGTCTCTGTACCAATACCATGC
NNNNNNNN
AGTTTTTACCACAATTGCTCTATAGTAAAGCTTGAGGTCAGGGTTGGTGATCCCTCCAGCCATTCTTTCATTATTAAGAA
TTGTTTTCCCTAGTCTGGGTTTTTTGCTTTTCCAGGCGAATTTGAGAATTGCTCTTTCCATGTCTTTGAAGAATTGTGTT
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
GGGATTTTGATGGGGTTTGCATTGAATCTGTAGATTGTCTTTGGTAAGATGGTTAGTTTTACTATGTTAATTCTGCCAAT
CCACAAGCATGGGAGCGCTCTCCATTTTCTGAGATCTTCTTCAATTTCTTTCTTGAGAAACTTGAAGTTATTGTCATACA
```

Number of Ns = predicted gap size,
with error bars (can’t be displayed in fasta format)

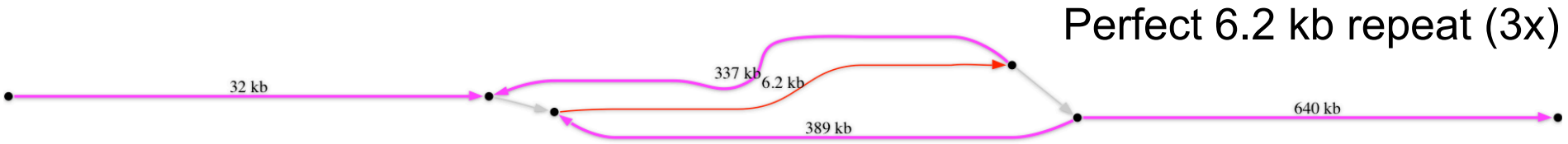
1. Linear assemblies

Example of an assembly in fasta format

```
>scaffold_1
TCCTAGATCCACTTGGACTTGAGCTTTGTACAAGATGACATATATAGGTCTGTTTTTATTCTTCTACATACAGACAGCCA
GTTATACCAGCACCATTTATTGAAGACACTTTCTTTATTCCATTGTATATTTTTTTACTTCCTTGTCAAAAATCAAGTGA
CCATGAGTATGTGGTTTCATTTCTGGGTCTTCAATTGTATTCCATTAGTCAACATATCTGTCTCTGTACCAATACCATGC
NNNNNNNN
AGTTTTTACCACAATTGCTCTATAGTAAAGCTTGAGGTCAGGGTTGGTGATCCCTCCAGCCATTCTTTCATTATTAAGAA
TTGTTTTCCCTAGTCTGGGTTTTTTGCTTTTCCAGGCGAATTTGAGAATTGCTCTTTCCATGTCTTTGAAGAATTGTGTT
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
GGGATTTTGATGGGGTTTGCATTGAATCTGTAGATTGTCTTTGGTAAGATGGTTAGTTTTACTATGTTAATTCTGCCAAT
CCACAAGCATGGGAGCGCTCTCCATTTTCTGAGATCTTCTTCAATTTCTTTCTTGAGAAACTTGAAGTTATTGTCATACA
>scaffold_2
CTGAAGTTGTTTATCAGCTGGAGAAGTTCTCAGGTAGAATTTTTGGGATTGCTTATGTATGCTATCATATCACTTGCAAA
TAGTGATACCTTGATTTCTTTTTTACCAATATGTATCCCATTGATCTCTTTCTGTTGTCTTATTGTTCTAGCTAACACTT
CAAGTACTATATTGAATAGATATGGGGAGAGTGGGAATCCTTGTCTTGTCTCCGATTTCAGTGGGATTGCTTCAAGTATG
```

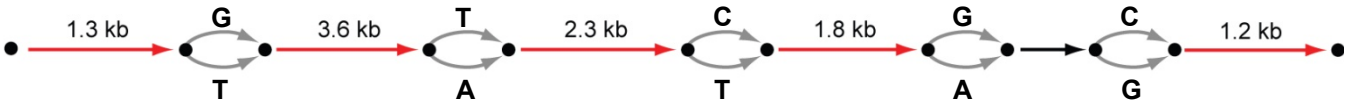
2. Graph assemblies

Graph assemblies are different from linear assemblies: they can have branches



Unresolved homopolymers

T^n, not sure what n is



SNPs

Graphs retain intrinsic ambiguity, allow alternatives
Downside: complicated!

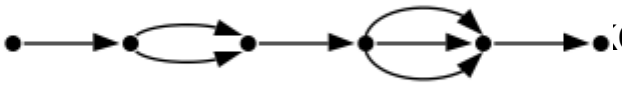
3. Linearized graph assemblies

Efasta

...ACTGTTT{A,C}GAAAT... A or C at site

...CGCGTTTTTTTTTT{,T,TT}CAT... 0 or 1 or 2 Ts at site

Can represent 'linear' graphs like this:

Linearized graph assembly: an assembly consisting of contigs in scaffolds, with e  e these

ALLPATHS-LG produces these.
Still figuring out the ideal solution….
Note challenge for community to figure out how to use!

3. Linearized graph assemblies

Example of an assembly in efasta format

```
>scaffold_1
TCCTAGATCCACTTGGACTTGAGCTTTGTATATATATATATATATATATA{,TA}CAAGATGACATATATAGGAGACAGCCA
GTTATACCAGCACCATTTATTGAAGACACTTTCTTTATTCCATTGTATATTTTTTTACTTCCTTGTCAAAAATCAAGTGA
CCATGAGTATGTGGTTTCATTTCTGGGTCTTCAATTGTATTCCATTAGTCAACATATCTGTCTCTGTACCAATACCATGC
NNNNNNNN
AGTTTTTACCACAATTGCTCTATAGTAAAGCTTGAGGTCAGGGTTGGTGATCCCTCCAGCCATTCTTTCATTATTAAGAA
TTGTTTTCCCTAGTCTGGGTTTTTTGCTTTTCCAGGCGAATTTGAGAATTGCTCTTTCCATGTCTTTGAAGAATTGTGTT
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
GGGATTTTGATGGGGTTTGCATTGAATCTGTAGATTGTCTTTGGTAAGATGGTTAGTTTTACTATGTTAATTCTGCCAAT
CCACAAGCATGGGAGCGCTCTCCATTTTCTGAGATCTTCTTCAATTTCTTTCTTGAGAAACTTGAAGTTATTGTCATACA
>scaffold_2
CTGAAGTTGTTTATCAGCTGGAGAAGTTCTCAGGTAGAATTTTTGGGATT{A,C,G}GCTTATGTATGCTATCTTGCAAA
TAGTGATACCTTGATTTCTTTTTTACCAATATGTATCCCATTGATCTCTTTCTGTTGTCTTATTGTTCTAGCTAACACTT
CAAGTACTATATTGAATAGATATGGGGAGAGTGGGAATCCTTGTCTTGTCTCCGATTTCAGTGGGATTGCTTCAAGTATG
```

How to use ALLPATHS-LG

1. What is an ALLPATHS-LG assembly?
2. **Data requirements (*** most critical thing ***)**
3. Computational requirements
4. Installation
5. Preparing your data
6. Assembling

ALLPATHS-LG sequencing model

Libraries (insert types)	Fragment size (bp)	Read length (bases)	Sequence coverage (x)	Required
Fragment	180*	≥ 100	45	yes
Short jump	3,000	≥ 100 preferable	45	yes
Long jump	6,000	≥ 100 preferable	5	no**
Fosmid jump	40,000	≥ 26	1	no**

*See next slide.

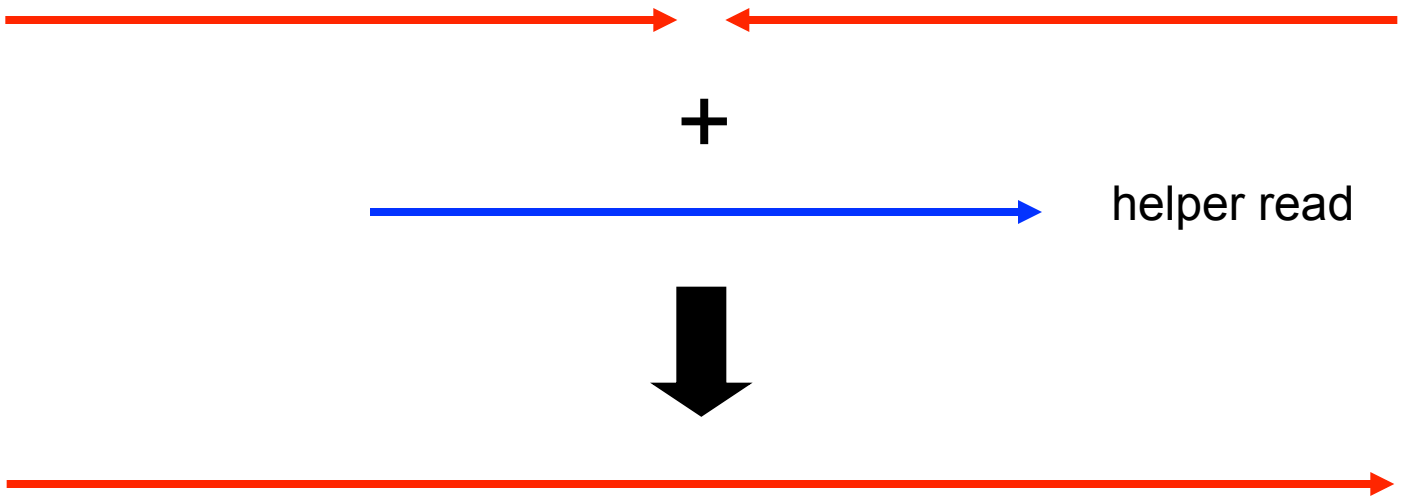
**For best results. Normally not used for small genomes. However essential to assemble long repeats or duplications.

Cutting coverage in half still works, with some reduction in quality of results.

All: protocols are either available, or in progress.

Libraries from 180 bp fragments

Pairs of 100 base reads from these libraries are merged to create ‘reads’ that are twice as long:

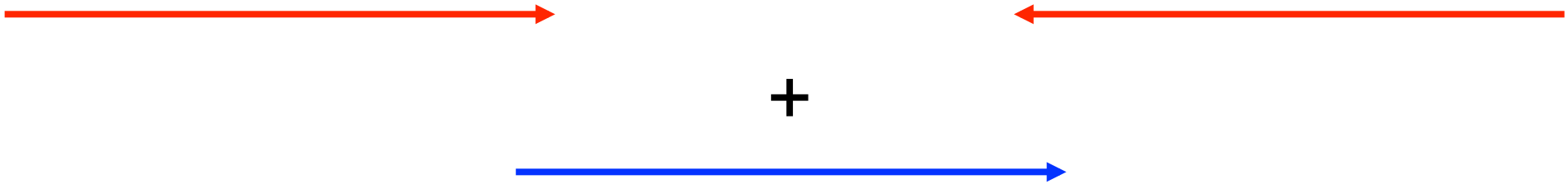


For longer reads, fragment size would be increased proportionally.

Fragment libraries

Potential problems:

(a) too many pairs too far apart

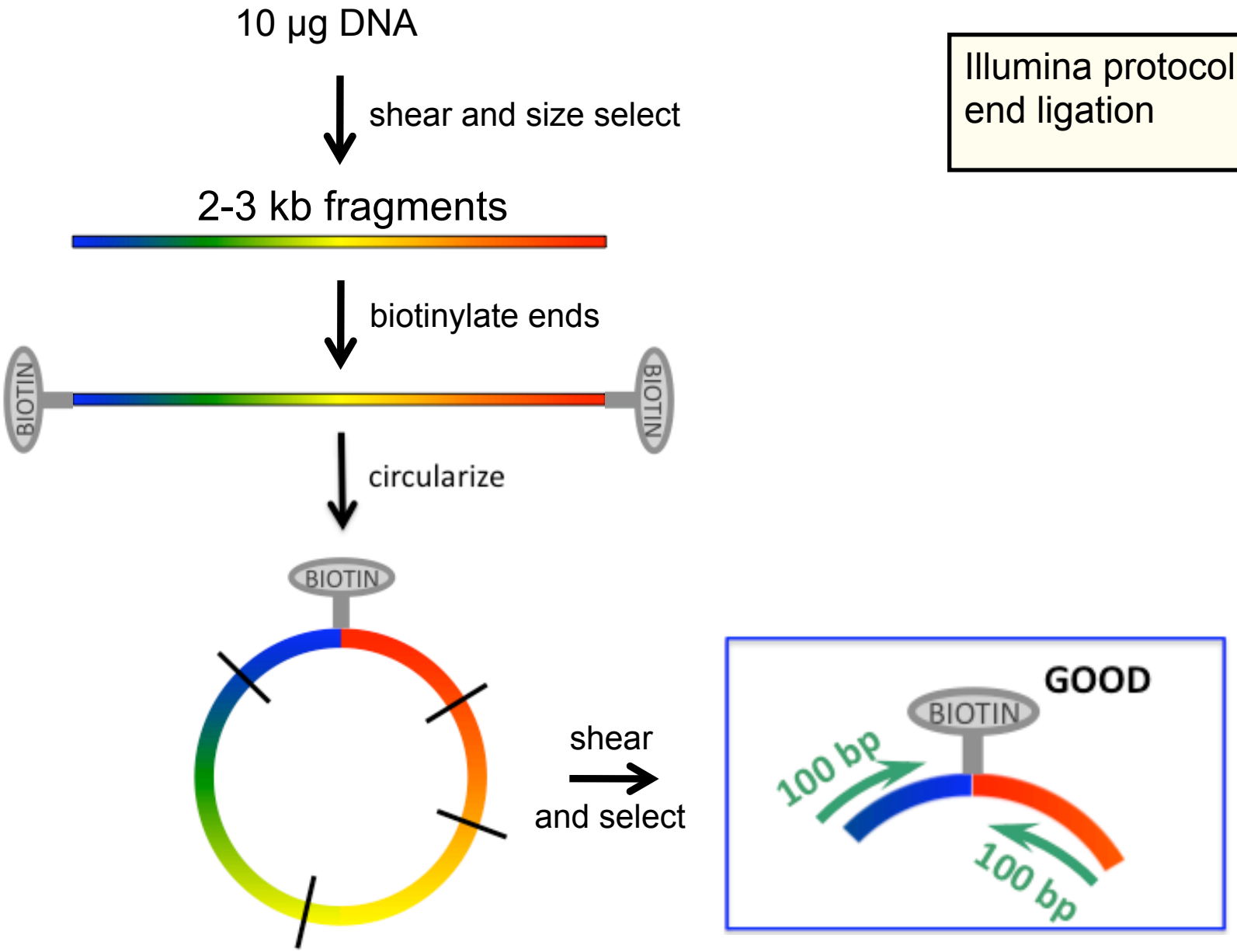


Consequence: these pairs lost by algorithm

(b) too many pairs too close together

Consequence: merged ‘reads’ too short, power lost

Short jumping libraries (2-3 kb)



Illumina protocol, blunt-end ligation

Short jumping libraries (2-3 kb)

Problem 1. Many steps → many opportunities for failure.

Example: a reagent might degrade. (This has happened.)

Short jumping libraries (2-3 kb)

Problem 2. Many steps → many DNA losses.

Here are *good* results for a mammalian genome:

Input: 10 µg DNA ⟷ ~3,000,000x physical coverage

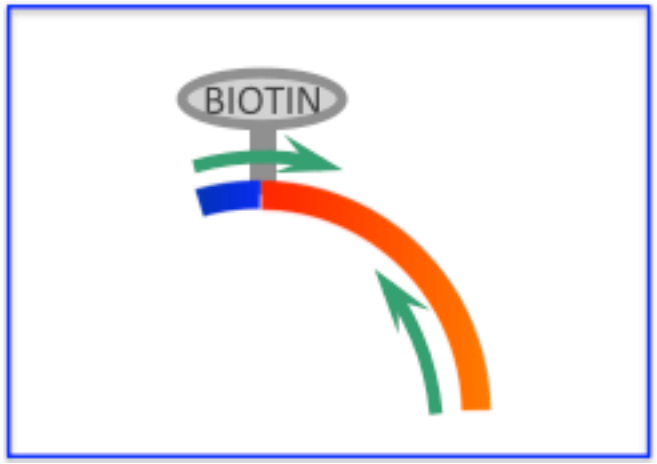
Output: (if fully sequenced) ~3,000x physical coverage

Loss: 99.9% (not including DNA between reads)

Small genomes are much easier!

Short jumping libraries (2-3 kb)

Problem 3. Read passes through circularization junction. This reduces the effective read length (and complicates algorithm).



What might be done to reduce incidence of this:
shear circles to larger size and select larger fragments

Short jumping libraries (2-3 kb)

Problem 4. Reads come from nonjumped fragments and are thus in reverse orientation and close together on the genome. This reduces yield (and complicates algorithm).

Putative cause: original DNA is nicked or becomes nicked during process – biotins become 'ectopically' attached at these nicks

Long jumping libraries (~6 kb)

Method 1. Instead of shearing circles, using EcoP15I restriction enzyme.

Pros
- demonstrated to work
- no artifacts

Cons
- read length = 26 bases

Method 2. Use Illumina blunt-end ligation protocol, but shear and size select larger fragments.

Pros
- long reads

Cons
- yield may be very low (probably not problem for small genomes)

How to use ALLPATHS-LG

1. What is an ALLPATHS-LG assembly?
2. Data requirements
3. **Computational requirements**
4. Installation
5. Preparing your data
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Computational requirements

- 64-bit Linux
- runs multi-threaded on a single machine
- memory requirements
  - about 160 bytes per genome base, implying
    - need 512 GB for mammal (Dell R315, 48 processors, $39,000)
    - need 1 GB for bacterium (theoretically)
  - if coverage different than recommended, adjust...
  - potential for reducing usage
- wall clock time to complete run
  - 5 Mb genome → 1 hour (8 processors)
  - 2500 Mb genome → 500 hours (48 processors)

Prerequisite software

- g++ compiler. We use version 4.4.3. Older versions may not work.
http://gcc.gnu.org

- C++ Boost library. We use version 1.39.
http://www.boost.org

- Picard command-line utilities for SAM file manipulation.
http://picard.sourceforge.net

How to use ALLPATHS-LG

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Installing ALLPATHS-LG

Web page:

http://www.broadinstitute.org/software/allpaths-lg/blog/

General instructions:

http://www.broadinstitute.org/science/programs/genome-biology/computational-rd/general-instructions-building-our-software

Getting the ALLPATHS-LG source

Our current system is to release code daily if it passes a test consisting of several small assemblies:

Download the latest build from:
ftp://ftp.broadinstitute.org/pub/crd/ALLPATHS/Release-LG/

Unpack it:

```
% tar xzf allpathslg-39099.tar.gz
```

(substitute the latest revision id for 39099)

This creates a source code directory `allpathslg-39099`:

```
% cd allpathslg-39099
```

Building ALLPATHS-LG

Step one: `./configure`

Options:
`-prefix=<prefix path>`
put binaries in `<prefix path>/bin`, else `./bin`
`--with-boost=<boost dir>`
you only need this if configure cannot find boost

Step two: `make` and `make install`

Options:
`-j<n>`
compile with n parallel threads

Step three: add bin directory to your path

How to use ALLPATHS-LG

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Preparing data for ALLPATHS-LG

Before assembling, prepare and import your read data.

ALLPATHS-LG expects reads from:

• At least one fragment library. One should come from fragments of size ~180 bp. This isn't checked but otherwise results will be bad.

• At least one jumping library.

IMPORTANT: use all the reads, including those that fail the Illumina purity filter (PF). These low quality reads may cover 'difficult' parts of the genome.

ALLPATHS-LG input format

ALLPATHS-LG can import data from:
BAM, FASTQ, FASTA/QUALA or FASTB/QUALB files.

You must also provide two metadata files to describe them:

`in_libs.csv` - describes the libraries
`in_groups.csv` - ties files to libraries

FASTQ format: consists of records of the form
@<read name>
<sequence of bases, multiple lines allowed>
+
<sequence of quality scores, with Qn represented by ASCII code n+33, multiple lines allowed>

Libraries – in_libs.csv (1 of 3)

`in_libs.csv` is a comma separated value (CSV) file.
For clarity, blanks and tabs are allowed and ignored.

The first line describes the field names, listed below.
Each subsequent line describes a library.

`library_name` - a unique name for the library.

Each physically different library should have a different name!

Libraries – in_libs.csv (2 of 3)

For fragment libraries only

`frag_size` - estimated mean fragment size

`frag_stddev` - estimated fragment size std dev

For jumping libraries only

`insert_size` - estimated jumping mean insert size

`insert_stddev` - estimated jumping insert size std dev

These values determine how a library is used. If `insert_size` is ≥ 20000, the library is assumed to be a Fosmid jumping library.

`paired` - always 1 (only supports paired reads)

`read_orientation` - `inward` or `outward`.

Paired reads can either point towards each other, or away from each other. Currently fragment reads must be `inward`, jumping reads `outward`, and Fosmid jumping reads `inward`.

Libraries – in_libs.csv (3 of 3)

Reads can be trimmed to remove non-genomic bases produced by the library construction method:

`genomic_start`
`genomic_end` - inclusive zero-based range of read bases to be kept; if blank or 0 keep all bases

Reads are trimmed in their original orientation.

Extra optional fields (descriptive only – ignored by ALLPATHS)

`project_name` - a string naming the project.
`organism_name` - the organism name.
`type` - fragment, jumping, EcoP15I, *etc*.

EXAMPLE

```
library_name,     type, paired, frag_size, frag_stddev, insert_size, insert_stddev, read_orientation, genomic_start, genomic_end
Solexa-11541, fragment,      1,       180,          10,           ,             ,           inward               ,
Solexa-11623,  jumping,      1,          ,           ,        3000,          500,          outward              0,          25
```

Input files – required format

Each BAM or FASTQ file contains paired reads from *one* library.

Data from a single library can be split between files.
Example, one file for each Illumina lane sequenced.

For FASTQ format, the paired reads can be divided in two files (`readsA.fastq`, `readsB.fastq`), or, if in a single file (`reads.fastq`), must be interleaved:

```
pair1_readA
pair1_readB
pair2_readA
pair2_readB
…
```

Input files – in_groups.csv

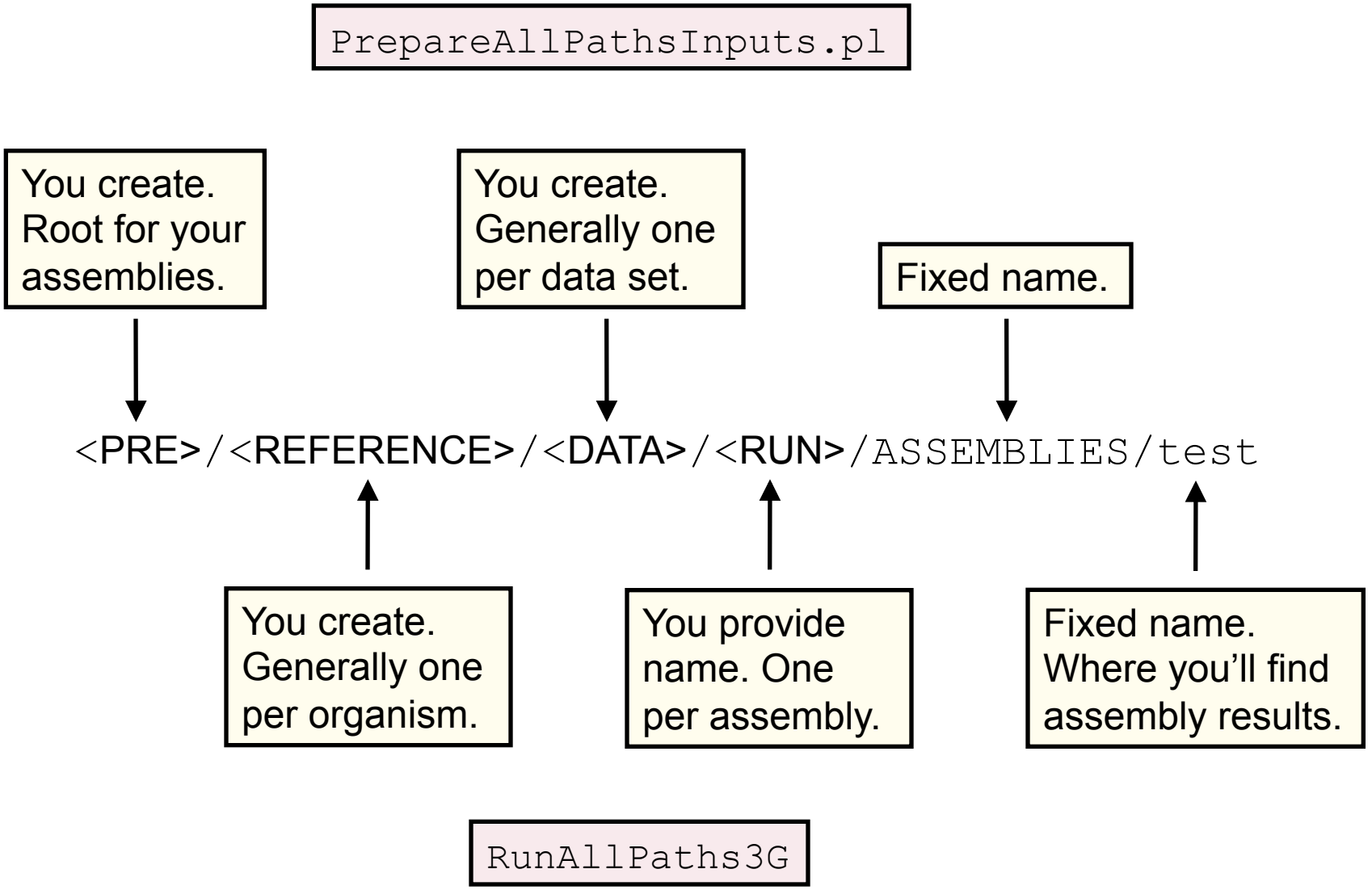
Each line in `in_groups.csv` comma separated value file, corresponds to a BAM or FASTQ file you wish to import for assembly.
The library name must match the names in `in_libs.csv`.

`group_name` - a unique nickname for this file
`library_name` - library to which the file belongs
`file_name` - the absolute path to the file
(should end in .bam or .fastq)
(use wildcards '?', '*' for paired fastqs)

Example:

```
group_name, library_name,                        file_name
302GJ,      Solexa-11541, /seq/Solexa-11541/302GJABXX.bam
303GJ,      Solexa-11623, /seq/Solexa-11623/303GJABXX.?.fastq
```

ALLPATHS-LG directory structure



How to import assembly data files

```
PrepareAllPathsInputs.pl
    IN_GROUPS_CSV=<in groups file>
    IN_LIBS_CSV=<in libs file>
    DATA_DIR=<full path of data directory>
    PLOIDY=<ploidy, either 1 or 2>
    PICARD_TOOLS_DIR=<picard tools directory>
    HOSTS=<list of hosts to be used in parallel>
```

- `IN_GROUPS_CSV` and `IN_LIBS_CSV`: optional arguments with default values `./in_groups.csv` and `./in_libs.csv`. These arguments determine where the data are found.

- `DATA_DIR`: imported data will be placed here.

(continued)

How to import assembly data files

- `PLOIDY`: either 1 (for a haploid or inbred organism), or 2 (for a diploid organism) – we have not tried to assemble organisms having higher ploidy!

- `PICARD_TOOLS_DIR`: path to Picard tools, for data conversion from BAM.

- `HOSTS`: optional list of hosts to use in parallel; example: `HOSTS="2,4.host3"`, translates to: 2 processes forked on this machine; 4 processes forked on host3. Forking to remote hosts requires password-less ssh access, *e.g.* using ssh-agent/ssh-add.

How to use ALLPATHS-LG

1. What is an ALLPATHS-LG assembly?
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How to assemble

Do this:

```
RunAllPathsLG                    \
   PRE=<prefix path>               \
   REFERENCE_NAME=<reference dir>    \
   DATA_SUBDIR=<data dir>              \
   RUN=<run dir>
```

Automatic resumption. If the pipeline crashes, fix the problem, then run the same `RunAllPathsLG` command again. Execution will resume where it left off.

Results. The assembly files are:

`final.contigs.fasta`	- fasta contigs
`final.contigs.efasta`	- efasta contigs
`final.assembly.fasta`	- scaffolded fasta
`final.assembly.efasta`	- scaffolded efasta

Putting it all together

1. Collect the BAM or FASTQ files that you wish to assemble. Create a `in_libs.csv` metadata file to describe your libraries and a `in_groups.csv` metadata file to describe your data files.

2. Create a data directory and import your data.

```
% mkdir -p /assemblies/E.coli/experiment1
% PrepareAllPathsInputs.pl                \
  DATA_DIR=/assemblies/E.coli/experiment1 \
  PICARD_TOOLS_DIR=<picard tools directory>  \
  PLOIDY=1
```

3. Assemble.

```
% RunAllPathsLG PRE=/assemblies                  \
  REFERENCE_NAME=E.coli          \
  DATA_SUBDIR=experiment RUN=assembly1
```

4. Get the results (four files).

```
  /assemblies/E.coli/experiment1/ASSEMBLIES/assembly1/
  final.{assembly,contigs}.{fasta,efasta}
```

Putting it all together

1. Prepare input files

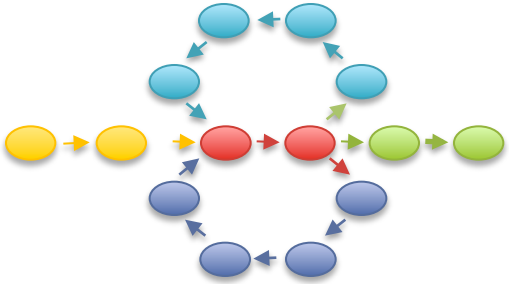
```
% cd /bluearc/data/schatz/mschatz/course/Data/original
% PrepareAllPathsInputs.pl \
   DATA_DIR=`pwd` \
   PICARD_TOOLS_DIR=~/build/packages/picard-tools-1.52/ \
   PLOIDY=1 GENOME_SIZE=5000000
```

2. Assemble.

```
% RunAllPathsLG \
  PRE=/bluearc/data/schatz/mschatz/course/ \
  DATA_SUBDIR=original RUN=default \
  REFERENCE_NAME=Data K=96 THREADS=24
```

3. Get the results (four files).

```
/assemblies/E.coli/experiment1/ASSEMBLIES/assembly1/
final.{assembly,contigs}.{fasta,efasta}
```



Running Quake + SOAPdenovo

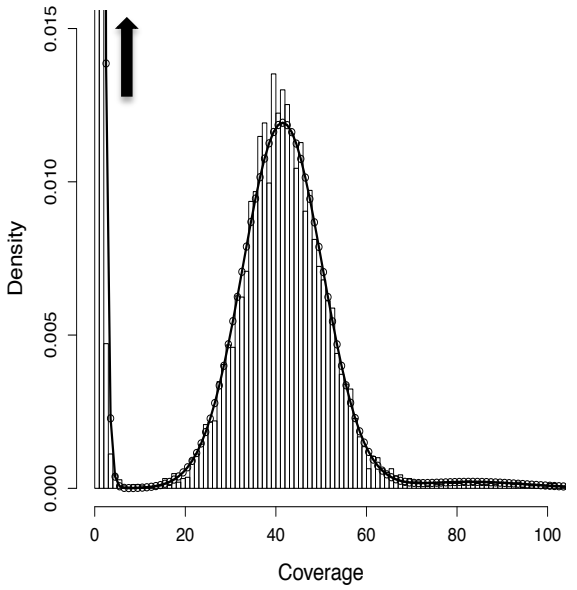
Quake

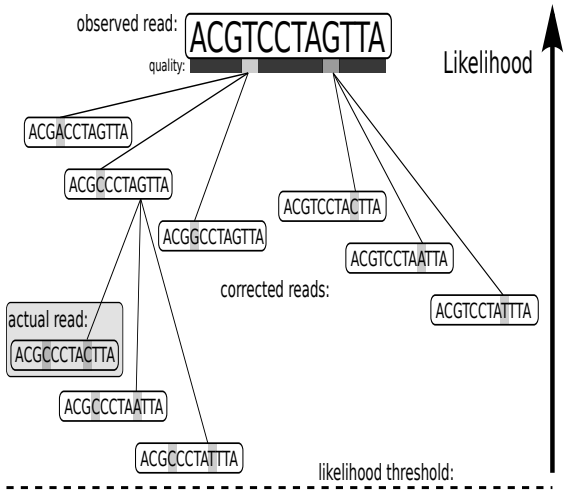
- Corrects substitution errors in Illumina reads by fitting kmers distribution to mixture model of erroneous/correct kmers

- Critical value is selecting appropriate k
  - Need to pick K so that k-mer expected to be unique in genome
    - For a 5 Mb genome set k ≥ 15
    - For a 3 Gb genome set k ≥ 19.
  - Quake requires more RAM as K gets larger
    - Requires 4^k bits of memory
    - 125Mb for k=15
    - 32GB for k=19

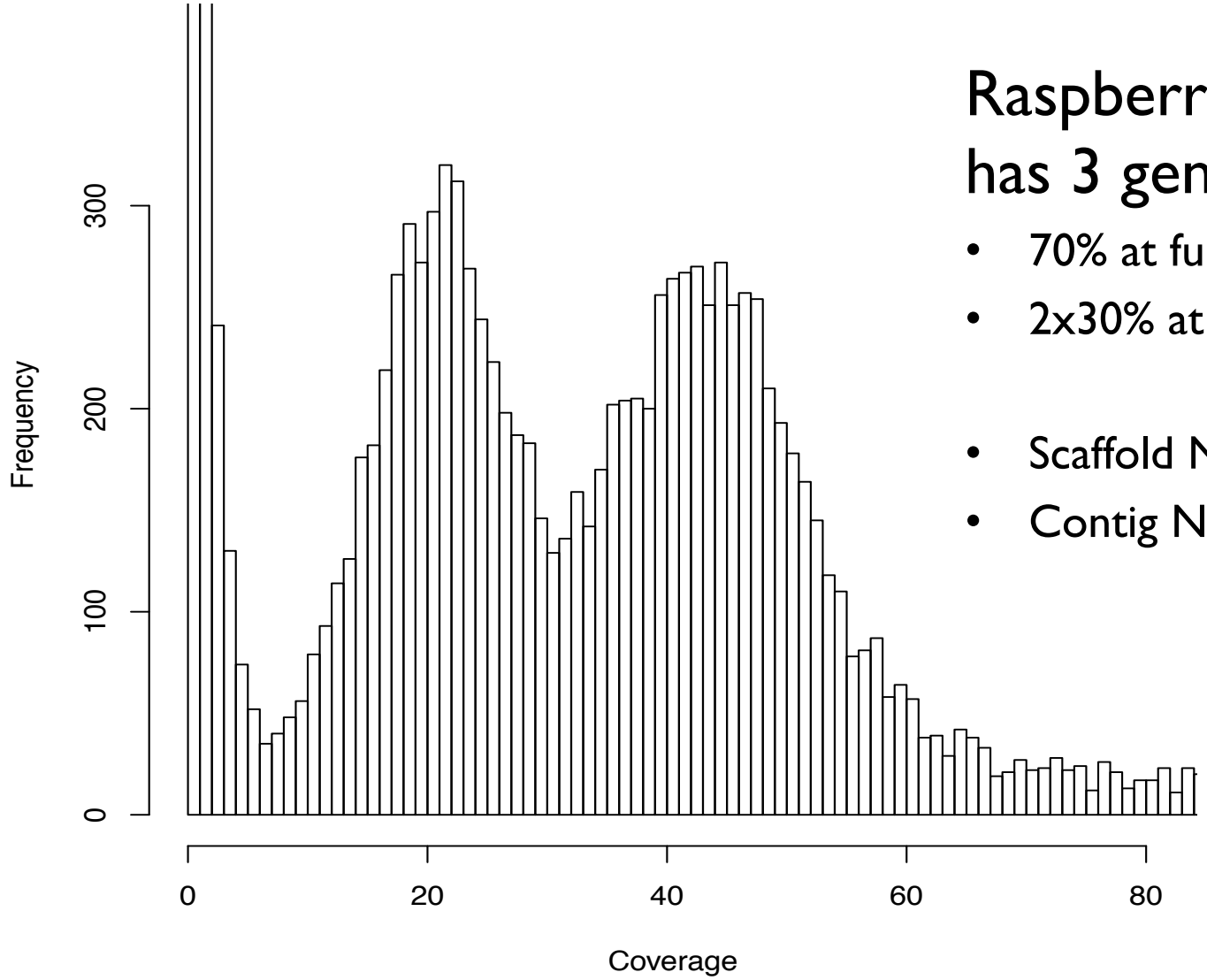
```
% cat > Data/original/quake_files.txt << EOF
frag_1.fastq frag_2.fastq
shortjump_1.fastq shortjump_2.fastq
EOF

% cd Data/original/ && ../../Quake/bin/quake.py
-f quake_files.txt -k 17 -p 20 --no_jelly
```





Heterozygous Genomes



Raspberry effectively has 3 genomes

- 70% at full coverage
- 2x30% at half coverage

- Scaffold N50: 17kbp
- Contig N50: 12kbp

```
## From kmers.txt
% Rscript Quake/bin/kmer_hist.r
```

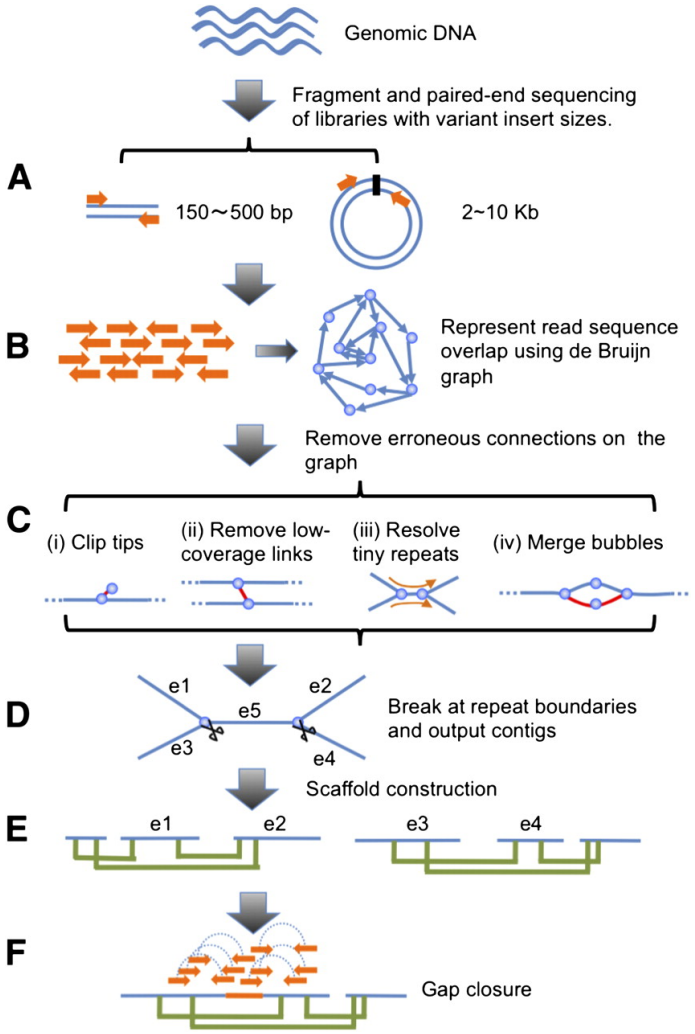
SOAPdenovo

Li *et al.* (2010) De novo assembly of human genomes with massively parallel short read sequencing. *Genome Research.* 20: 265-272

- Designed specifically for Illumina reads
- Constructs de Bruijn graph with varying k-mer size
  - 31-mer, 63-mer, and 127-mer versions available

- 4 major phases:
  - pregraph (B): construct de Bruijn graph
  - contig (C): correct errors
  - map: (D) realign reads to initial contigs
  - scaff: (D,E) resolve repeats, and errors

```
% SOAPdenovo63mer all -K 31 \
  -p 16 -s SOAPdenovo.config \
  -o asm >> SOAPdenovo.log
```



SOAPdenovo

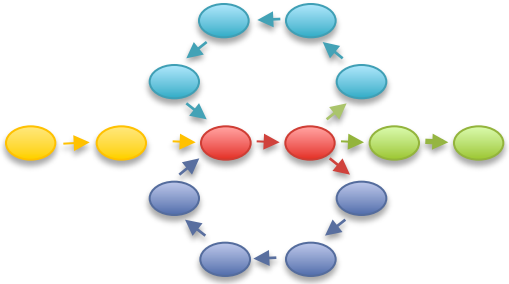
An example configuration file:

```
[LIB]
avg_ins=180
reverse_seq=0
asm_flags=1
rank=1
q1=../Data/original/frag_1.cor.fastq
q2=../Data/original/frag_2.cor.fastq

[LIB]
avg_ins=3500
reverse_seq=1
asm_flags=2
rank=2
q1=../Data/original/shortjump_1.cor.fastq
q2=../Data/original/shortjump_2.cor.fastq
```

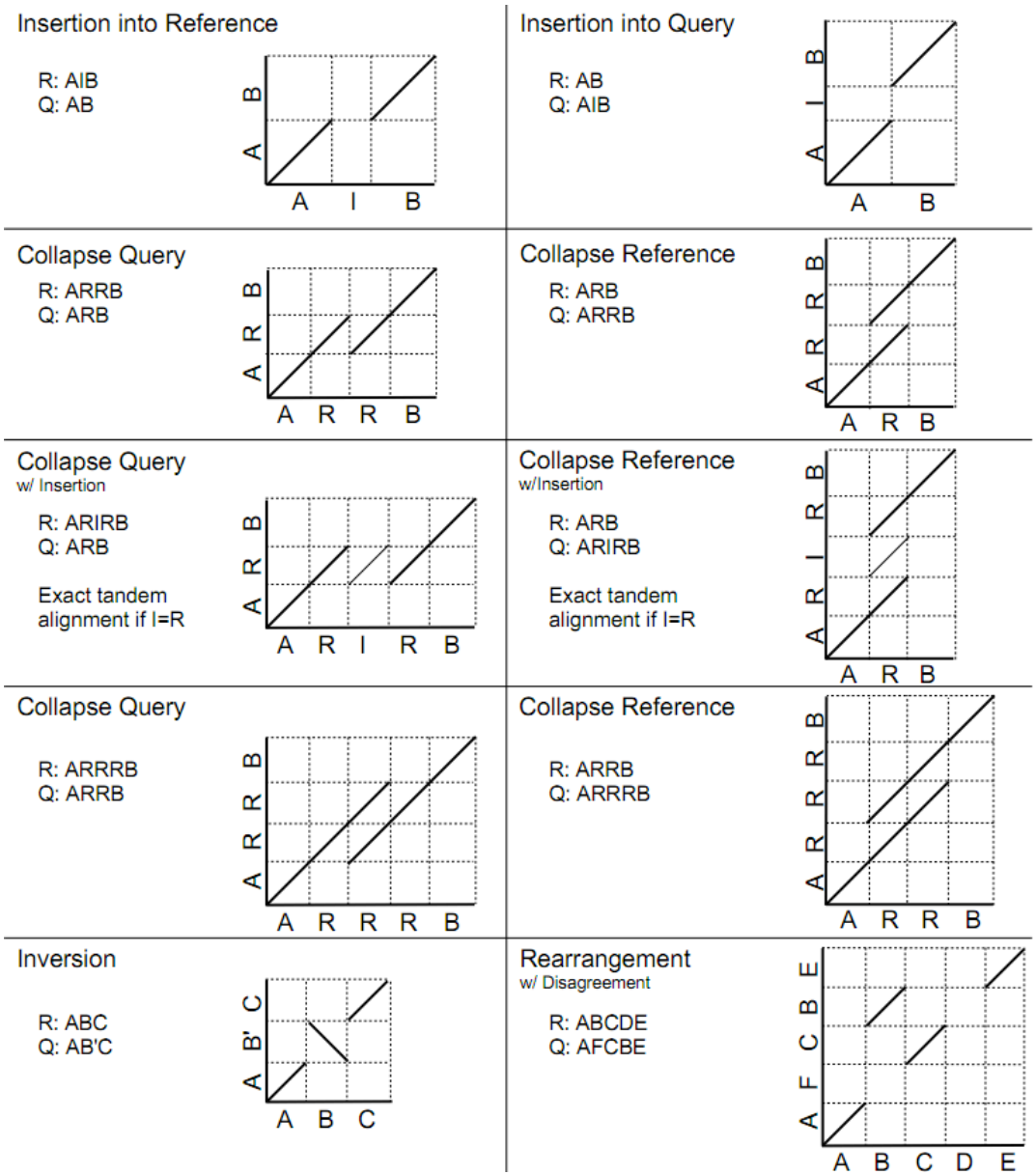
SOAPdenovo tips & tricks

- Input
  - Proper choice of K
  - Good error correction go reads to simplify SOAPdenovo's de Bruijn graph
- Execution
  - Can use different sets of reads for contig construction and for assembly
  - Simple and straightforward configuration file
  - Very fast, but high peak memory usage
- Output
  - asm.contig: initial contigs *before* scaffolding
  - asm.scafSeq: linear scaffolds
  - SOAPdenovo.log: Lots of status messages, intermediate files



Whole Genome Alignment with MUMmer

SV Types



- Different structural variation types / misassemblies will be apparent by their pattern of breakpoints
- Most breakpoints will be at or near repeats
- Things quickly get complicated in real genomes

http://mummer.sf.net/manual/AlignmentTypes.pdf

WGA Alignment

nucmer –maxmatch ref.fasta qry.fasta

```
-maxmatch    Find maximal exact matches (MEMs)
```

delta-filter –m out.delta > out.filter.m

```
-m   Many-to-many mapping
```

show-coords -rcl out.delta.m > out.coords

```
-r   Sort alignments by reference position
-c   Show percent coverage
-l   Show sequence lengths
```

show-aligns -rcl out.delta.m REFID QRYID > out.aligns

dnadiff out.delta.m

```
Construct catalog of sequence variations
```

mummerplot --large --layout out.delta.m

```
--large      Large plot
--layout Nice layout for multi-fasta files
--x11        Default, draw using x11 (--postscript, --png)
*requires gnuplot
```

See manual at http://
mummer.sourceforge.net/manual

Resources

- Assembly Competitions
  - Assemblathon: http://assemblathon.org/
  - GAGE: http://gage.cbcb.umd.edu/
- Assembler Websites:
  - ALLPATHS-LG: http://www.broadinstitute.org/software/allpaths-lg/blog/
  - SOAPdenovo: http://soap.genomics.org.cn/soapdenovo.html
  - Celera Assembler: http://wgs-assembler.sf.net
- Tools:
  - MUMmer: http://mummer.sourceforge.net/
  - Quake: http://www.cbcb.umd.edu/software/quake/
  - AMOS: http://amos.sf.net

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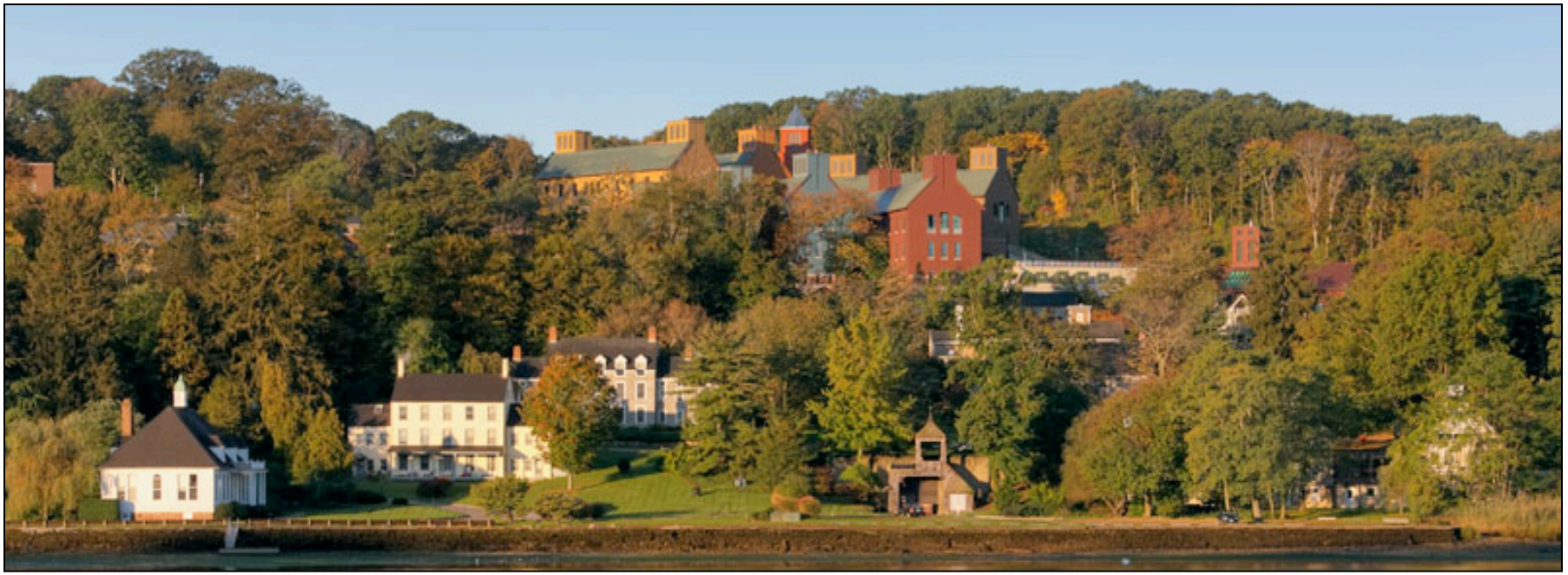

Thank You

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