De novo assembly of complex genomes
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Weill Cornell Medical College
Outline

1. Genome assembly by analogy
2. Hybrid error correction and assembly
3. De novo mutations in autism
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3. De novo mutations in autism
Shredded Book Reconstruction

• Dickens accidentally shreds the first printing of *A Tale of Two Cities*
  - Text printed on 5 long spools

  It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, ...
  It was the worst of times, it was the best of times, it was the age of wisdom, it was the age of foolishness, ...
  It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, ...
  It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, ...
  It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, ...

• How can he reconstruct the text?
  - 5 copies × 138,656 words / 5 words per fragment = 138k fragments
  - The short fragments from every copy are mixed together
  - Some fragments are identical
Greedy Reconstruction

The repeated sequence make the correct reconstruction ambiguous

- *It was the best of times, it was the [worst/age]*

Model sequence reconstruction as a graph problem.
de Bruijn Graph Construction

- $G_k = (V,E)$
  - $V =$ All length-$k$ subfragments ($k < l$)
  - $E =$ Directed edges between consecutive subfragments
    - Nodes overlap by $k-1$ words

Original Fragment | Directed Edge
--- | ---
It was the best of | It was the best \rightarrow was the best of

- Locally constructed graph reveals the global sequence structure
  - Overlaps between sequences implicitly computed

de Bruijn, 1946
Idury and Waterman, 1995
Pevzner, Tang, Waterman, 2001
It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness.

After graph construction, try to simplify the graph as much as possible.
It was the best of times, it was the age of foolishness. It was the worst of times, it was the age of wisdom.

After graph construction, try to simplify the graph as much as possible.
The full tale

... it was the best of times it was the worst of times ...
... it was the age of wisdom it was the age of foolishness ...
... it was the epoch of belief it was the epoch of incredulity ...
... it was the season of light it was the season of darkness ...
... it was the spring of hope it was the winter of despair …
N50 size

Def: 50% of the genome is in contigs as large as the N50 value

Example: 1 Mbp genome

\[
\text{N50 size} = 30 \text{ kbp} \\
(300k + 100k + 45k + 45k + 30k = 520k \geq 500kbp)
\]

Note:
A “good” N50 size is a moving target relative to other recent publications. 10-20kbp contig N50 is currently a typical value for most “simple” genomes.
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Assembly Applications

Novel genomes

Metagenomes

Sequencing assays
- Transcript assembly
- Structural variations
- Haplotype analysis
- …
Ingredients for a good assembly

Read Length

Reads & mates must be longer than the repeats
- Short reads will have false overlaps forming hairball assembly graphs
- With long enough reads, assemble entire chromosomes into contigs

Coverage

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

Quality

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

Current challenges in de novo plant genome sequencing and assembly
Hybrid Sequencing

**Illumina**
*Sequencing by Synthesis*

- High throughput (60Gbp/day)
- High accuracy (~99%)
- Short reads (~100bp)

**Pacific Biosciences**
*SMRT Sequencing*

- Lower throughput (1Gbp/day)
- Lower accuracy (~85%)
- Long reads (5kbp+)
SMRT Sequencing

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

SMRT Read Types

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

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

Sample of 100k reads aligned with BLASR requiring >100bp alignment

<table>
<thead>
<tr>
<th>Match</th>
<th>83.7%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insertions</td>
<td>11.5%</td>
</tr>
<tr>
<td>Deletions</td>
<td>3.4%</td>
</tr>
<tr>
<td>Mismatch</td>
<td>1.4%</td>
</tr>
</tbody>
</table>
1. Correction Pipeline
   1. Map short reads to long reads
   2. Trim long reads at coverage gaps
   3. Compute consensus for each long read

2. Error corrected reads can be easily assembled, aligned

Hybrid error correction and de novo assembly of single-molecule sequencing reads.
Enhanced PacBio Error Correction

https://github.com/jgurtowski/pbtools

PacBioToCA fails in complex regions

1. Simple Repeats – Kmer Frequency Too High to Seed Overlaps
2. GC Rich Regions – Known Illumina Bias
3. Error Dense Regions – Difficult to compute overlaps with many errors
Correction with Unitigs

Unitigs:
High quality contigs formed from unambiguous, unique overlaps of reads

Illumina reads ->
  Illumina unitigs ->
    Map and error correct PacBio reads ->
      Assemble PacBio reads

Can Help us overcome:
1. **Simple Repeats** – Kmer Frequency Too High to Seed Overlaps
2. GC Rich Regions – Known Illumina Bias
3. **Error Dense Regions** – Difficult to compute overlaps with many errors
Population structure in *Oryza sativa*

3 varieties selected for *de novo* sequencing

- High quality BAC-by-BAC reference
  - ~370 Mbp genome in 12 chromosomes
  - About 40% repeats:
    - Many 4-8kbp repeats
    - 300kbp max high identity repeat (99.99%)
  - Useful model for other cereal genomes

Garris et al. (2005)
*Genetics* 169: 1631–1638
PacBio Long Read Rice Sequencing

C1 Chemistry – Summer 2011
Median=639 Mean=824 Max=10,008

C2XL Chemistry – Summer 2012
Median=2231 Mean=3290 Max=24,405
Preliminary Rice Assemblies

<table>
<thead>
<tr>
<th>Assembly</th>
<th>Contig NG50</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiSeq Fragments</td>
<td>3,925</td>
</tr>
<tr>
<td>50x 2x100bp @ 180</td>
<td></td>
</tr>
<tr>
<td>MiSeq Fragments</td>
<td>6,332</td>
</tr>
<tr>
<td>23x 459bp</td>
<td></td>
</tr>
<tr>
<td>8x 2x251bp @ 450</td>
<td></td>
</tr>
<tr>
<td>“ALLPATHS-recipe”</td>
<td>18,248</td>
</tr>
<tr>
<td>50x 2x100bp @ 180</td>
<td></td>
</tr>
<tr>
<td>36x 2x50bp @ 2100</td>
<td></td>
</tr>
<tr>
<td>51x 2x50bp @ 4800</td>
<td></td>
</tr>
</tbody>
</table>

In collaboration with McCombie & Ware labs @ CSHL
Improved Gene Reconstruction

FOXP2 assembled in a single contig in the PacBio parrot assembly

Hybrid error correction and de novo assembly of single-molecule sequencing reads.
Transcript Alignment

- Long-read single-molecule sequencing has potential to directly sequence full length transcripts
  - Raw reads and raw alignments (red) have many spurious indels inducing false frameshifts and other artifacts
  - Error corrected reads almost perfectly match the genome, pinpointing splice sites, identifying alternative splicing
- New collaboration with Gingeras Lab looking at splicing in human

Hybrid error correction and de novo assembly of single-molecule sequencing reads.
Internal Roadmap has made steady progress towards improving read length and throughput.

Very recent improvements:
1. Improved enzyme:
   Maintains reactions longer

2. “Hot Start” technology:
   Maximize subreads

3. MagBead loading:
   Load longest fragments
Simulate PacBio-like reads to predict how the assembly will improve as we add additional coverage.

Only 8x coverage is needed to sequence every base in the genome, but 40x improves the chances repeats will be spanned by the longest reads.

Assembly complexity of long read sequencing
Lee, H, Gurtowski, J, Marcus, S., Schatz MC et al. (2013) In preparation
Speculation for 2013

Doubling the average read length dramatically improves the assembly quality
• Able to span a larger repeats and lock contigs together

Expect to see contig N50 values over 1Mbp very soon, even in very complicated plant and animal species
• Megabase contig N50 already routine in microbial assembly with PacBio sequencing
With PacBio-like reads averaging 11.2kbp (4x current), we should be able to assemble almost every chromosome arm of rice into single contigs

- The 300kbp near perfect repeat is the only exception

Even with the current assembly, we are seeing new genes and other sequences missing in the “high quality” BAC-by-BAC reference genome.
Assembly Complexity of Long Reads
De novo assembly of Arabidopsis


A. thaliana Ler-0 sequenced at PacBio

- Sequenced using the latest P4 enzyme and C2 chemistry
- Size selection using an 8 Kb to 50 Kb elution window on a BluePippin™ device from Sage Science
- Total coverage >100x

Genome size: 124.6 Mb
GC content: 33.92%
Raw data: 11 Gb
Assembly coverage: 15x over 9kbp

Sum of Contig Lengths: 149.5 Mb
Number of Contigs: 1788
Max Contig Length: 12.4 Mb
N50 Contig Length: 8.4 Mb
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Variation Detection Complexity

SNPs + Short Indels
High precision and sensitivity

```
|   |   |   |   |   |
AGAATAGGC CGAG
```

“Long” Indels (>5bp)
Reduced precision and sensitivity

```
|   |   |   |   |   |   |
TT TAG A A T A G G C C |   |   |
TT TAG A A T A G G C C
```

Analysis confounded by sequencing errors, localized repeats, allele biases, and mismapped reads
Scalpel: Haplotype Microassembly

DNA sequence **micro-assembly** pipeline for accurate detection and validation of *de novo* mutations (SNPs, indels) within exome-capture data.

Features

1. Combine **mapping** and **assembly**

2. Exhaustive search of **haplotypes**

3. **De novo** mutations

NRXN1 *de novo* SNP
(auSSC12501 chr2:50724605)
Scalpel Pipeline

Extract reads mapping within the exon including (1) well-mapped reads, (2) soft-clipped reads, and (3) anchored pairs

Decompose reads into overlapping $k$-mers and construct de Bruijn graph from the reads

Find end-to-end haplotype paths spanning the region

Align assembled sequences to reference to detect mutations

deletion insertion
Experimental Analysis & Validation

Selected one deep coverage exome for deep analysis
- 80% of the target at >20x coverage
- Evaluated with Scalpel, SOAPindel, and GATK Haplotype Caller

1000 indels selected for validation
- 200 Scalpel
- 200 GATK Haplotype Caller
- 200 SOAPindel
- 200 within the intersection
- 200 long indels (>30bp)
Scalpel Indel Discovery

Detection of de novo mutations in exome-capture data using micro-assembly
Narzisi et al. (2013) In preparation
Scalpel Indel Discovery

Detection of de novo mutations in exome-capture data using micro-assembly
Narzisi et al. (2013) In preparation
Detection of de novo mutations in exome-capture data using micro-assembly
Narzisi et al. (2013) *In preparation*
Detection of de novo mutations in exome-capture data using micro-assembly
Narzisi et al. (2013) In preparation
Exome sequencing of the SSC

Sequencing of 343 families from the Simons Simplex Collection
- Parents plus one child with autism and one non-autistic sibling
- Enriched for higher-functioning individuals

Families prepared and captured together to minimize batch effects
- Exome-capture performed with NimbleGen SeqCap EZ Exome v2.0 targeting 36 Mb of the genome.
- ~80% of the target at >20x coverage with ~93bp reads

De novo gene disruptions in children on the autism spectrum
De novo mutation discovery and validation

**Concept:** Identify mutations not present in parents.

**Challenge:** Sequencing errors in the child or low coverage in parents lead to false positive de novos

Ref: \[\ldots TCAGAACAGCTGGATGAGATCTTAGCCAACCTACCAGGAGATTGTCTTTGCCCNGA\ldots \]

**Father:** \[\ldots TCAGAACAGCTGGATGAGATCTTAGCCAACCTACCAGGAGATTGTCTTTGCCCNGA\ldots \]

**Mother:** \[\ldots TCAGAACAGCTGGATGAGATCTTAGCCAACCTACCAGGAGATTGTCTTTGCCCNGA\ldots \]

**Sib:** \[\ldots TCAGAACAGCTGGATGAGATCTTAGCCAACCTACCAGGAGATTGTCTTTGCCCNGA\ldots \]

**Aut(1):** \[\ldots TCAGAACAGCTGGATGAGATCTTAGCCAACCTACCAGGAGATTGTCTTTGCCCNGA\ldots \]

**Aut(2):** \[\ldots TCAGAACAGCTGGATGAGATCTTAGCCAACCTACCAGGAGATTGTCTTTGCCCNGA\ldots \]

6bp heterozygous deletion at chr13:25280526 ATP12A
De novo Genetics of Autism

• In 343 family quads so far, we see significant enrichment in de novo likely gene killers in the autistic kids
  – Overall rate basically 1:1 (432:396)
  – 2:1 enrichment in nonsense mutations
  – 2:1 enrichment in frameshift indels
  – 4:1 enrichment in splice-site mutations
  – Most de novo originate in the paternal line in an age-dependent manner (56:18 of the mutations that we could determine)

• Observe strong overlap with the 842 genes known to be associated with fragile X protein FMPR
  – Related to neuron development and synaptic plasticity
  – Also strong overlap with chromatin remodelers

De novo gene disruptions in children on the autism spectrum
Summary

• Hybrid assembly let us combine the best characteristics of 2\textsuperscript{nd} and 3\textsuperscript{rd} gen sequencing
  – Long reads and good coverage are the keys to a good de novo assembly
  – Single contig de novo assemblies of entire microbial chromosomes are now routine; Single contig de novo assemblies of entire plant and animal chromosomes on the horizon

• Assembly is the missing link towards high accuracy indel mutation discovery
  – Allows the algorithm to break free from the expectations of the reference
  – Pinpointing de novo mutations require both high sensitivity and specificity

• We are starting to apply these technologies to discover significant biology that is otherwise impossible to measure
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