Single-Molecule Real-Time and Single-Cell RNA Sequencing

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• Introduction
  • Genome, Transcriptome, Sequencing and Challenges

• Single-Molecule Real-Time Sequencing
  • Requirements, experimental setup and obtained data

• Single-Cell whole Transcriptome Sequencing
  • Transcriptome analysis, preparation from tissue to cDNA library

• Conclusion and Outlook
• Determination of primary structures of biopolymers

• Provides linear sequence of sub-structures

• Summarized much about atomic-level structure

• Especially interesting for DNA and RNA
• Storage of genetic information of all living organisms
• Built-up from monomeric units → nucleotides
• Certain sequences of nucleotides encode genes
• Genes further encode e.g. proteins

Gene expression:

- Transcription: DNA → RNA
- Translation: RNA → Protein

Genotype: Genetic composition of an Organism

Phenotype: Observable appearance of an Organism
Limitation of previous techniques

- **Sanger method**
  - uses end tags and gel-electrophoresis for analysis
  - Longer strands sequencing possible, but very long read times

- **Alternative approaches (2\textsuperscript{nd} generation sequencing):**
  - Faster overall throughput
  - Sequence reads limited to \(~400\) nucleotides or shorter (e.g. Illumina)

- **Often huge quantities are required for analysis**
Single-Molecule Real-Time Sequencing
Idea: DNA polymerase as real-time sequencing machine

fast, long reads

Polymerase integrates a nucleotide.

Hydrogen and pyrophosphate are released.

Phospholinked fluorescent nucleotides

- Different colors to distinguish different nucleotides
- Fluorescent dyes are released during incorporation of nucleotide

natural DNA

Pacific Biosciences Technology Backgrounder (11/24/2008)
Zero-Mode Waveguide (ZMW)

- Sub-wavelength aperture
- Observation volume confinement in zeptoliter ($10^{-21}$) regime

Schematic of single ZMW

Intensity distribution inside a ZMW

• DNA polymerase immobilized at bottom of ZMW, synthesizing DNA
• Incorporation of fluorescently labeled nucleotides produces light pulse
• Record fluorescent signals over time

**Figure 13. Highly parallel optics system.**

The detected flash of light is separated into a spatial array, from which the identity of the incorporated base is determined.
Fluorescence time trace on linear template with 150 bases

Properties of SMRT sequencing

- Long read lengths (possible up to > 30kbp)
- Short run times (~ 5 bases/s)
- No amplification needed

Real-time video of single molecule DNA polymerase activity measured on an array of 3,000 ZMWs.

http://science.sciencemag.org/content/suppl/2008/11/20/1162986.DC1 (02.12.2018)
Single cell sequencing

scRNA-seq method for whole transcriptome sequencing

(based on Tang et al., 2009)
Steps of scRNA-seq

1) Isolation and lysis of single cells or single nuclei

2) Reverse transcription

3) cDNA amplification

4) Sequencing library preparation

5) Application of single-molecule sequencing
Hedlund, Deng (2018)
• Synthesis cDNA from mature mRNA
• Using poly-T primer with anchor sequence (UP1)
• Digestion of unused Primers
• Addition of poly-A tail to 3’-end of first cDNA strand
• Synthesis of the second cDNA strand
• Using again poly-T primers, but different anchor sequence (UP2)

Tang et al. (2009)
Amplification of cDNA

- Multiplexing of cDNA using PCR
- Anchor sequences as primers for DNA-Polymerase

Tang et al. (2009)
• Analysis of rare cell types and subpopulations
• Study of infectious diseases
• Characterisation of tumour cells
Single Cell RNA- Sequencing (scRNA-seq):

- Direct Sequencing of RNA not possible
- Reversed Transcription from RNA to cDNA
- cDNA Amplification and Analysis Sequencing methods
- Increase throughput and accuracy
- Non-adenylation dependent methods

Single-Molecule Real-Time Sequencing (SMRT):

- Observing polymerase during DNA synthesis

Possible due to:
- Phospholinked fluorescent nucleotides
- Zero-Mode Waveguide
• Long read lengths
• Short run times
• No amplification needed

Applications:
• Whole genome sequencing
• De Novo sequencing
• Monitor polymerase dynamics
• Single-Cell RNA sequencing
• ....
Thanks for your Attention!
• Pacific Biosciences Technology Backgrounder (11/24/2008)
  https://www.ndsu.edu/pubweb/~mcclean/plsc411/Pacific%20Biosciences-technology_backgrounder.pdf