Fluorescence *in situ* Hybridization (FISH)  
“Fishing” in the Genomic Wilderness

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Clinical Assistant Professor, University of British Columbia
Overview

1. The Chromosomes and Cell Cycle
2. A Brief History of Classical Cytogenetics
3. FISH – the nuts and bolts
4. Clinical FISH – Application in:
   – Pre-natal Genetics
   – Constitutional Genetics
   – Cancer Genetics
ATOMS  MOLECULES  ORGANELLES  CELLS  TISSUES

http://www.nature.com/scitable/content/the-relative-scale-of-biological-molecules-and-14704956
1. At the simplest level, chromatin is a double-stranded helical structure of DNA.

2. DNA is complexed with histones to form nucleosomes.

3. Each nucleosome consists of eight histone proteins around which the DNA wraps 1.65 times.

4. A chromosome consists of a nucleosome plus the H1 histone.

5. The nucleosomes fold up to produce a 30-nm fiber...

6. ...that forms loops averaging 300 nm in length.

7. The 300-nm fibers are compressed and folded to produce a 250-nm-wide fiber.

8. Tight coiling of the 250-nm fiber produces the chromatid of a chromosome.
Excess Thymidine

Colcemid/Colchicine

G0 = most cells

STIMULATED
PHA

Pokeweed

T-cells

T-cells and B-cells
Dude, mitosis starts in five minutes... I can’t believe you’re not condensed yet.
A normal diploid human cell has how many chromosomes?

a) 23
b) 44
c) 46
d) 48
A normal diploid human cell has how many chromosomes (circa 1928-1950)?

- a) 23
- b) 44
- c) 46
- d) 48

In 1928 T.S. Painter published that humans have 48 chromosomes and established Y chromosome as mechanism of sex determination. It wasn’t until 1956 that the correct number of 46 was reported (Tijo and Levan, 1946).
Key Cytogenetic Discoveries of the 20th Century

• Hypotonic treatment of cells prior to fixation to swell nuclei

1953 – T.C. Hsu
Key Cytogenetic Discoveries of the 20th Century

- Banding Techniques for staining chromosomes to they could be identified
  - Q-bands
  - G-bands
  - R-bands
  - C-bands
  - Ag-Nor staining
Key Cytogenetic Discoveries of the 20th Century

- 1990 – Fluorescence in situ hybridization
- 1996 – Multicolour FISH (or SKY)
- 2001 – Cytogenetic “chips” – microrrays of DNA probes that can detect genomic gains and losses.
Life’s Lessons

• 3 essential ingredients in French cooking...
  – Butter
  – Butter
  – Butter

• 3 rules for a happy marriage...
  – She’s the boss
  – She’s the boss
  – She’s the boss

• 3 concepts for a complete understanding of molecular biology
  – Hybridization
  – Hybridization
  – Hybridization
Fluorescence in situ hybridization (FISH):

- DNA probes are labeled by incorporation of chemically modified nucleotides that fluoresce directly (direct) or can be detected by binding a fluorescently tagged reporter molecule (indirect)
- these single stranded probes are hybridized to metaphase chromosomes, or interphase nuclei
- analyzed using a fluorescent microscope
Once nucleation has occurred, renaturation is rapid due to zippering.

As the temperature is increased, the bases unstack, and hydrogen bonds break.

At higher temperatures, even G/C-rich regions are disrupted.

When the solution is returned to lower temperatures, complementary regions on separate strands form base pairs.

Area of nucleation

Partially unwind DNA

Area rich in A/T base pairs

Totally denatured DNA (separate strands)
Fluorescence In Situ Hybridization

- Probe DNA
- Labeling with fluorescent dye
- Denature & Hybridize
- Chromosome with fluorescent signal
Overview of Cytogenetic Procedure - FISH

Blood collected in sodium heparin

1. Drop on Slides
2. Denature
3. Hybridize probe to slide

Digital capture & analysis

Fluorescent Microscope analysis

Add colchicine (metaphase arrest)

Hypotonic solution; Fixative
How to make a FISH probe?

1. *Buy it*
2. *BAC*
3. Fosmid/PAC/YAC
4. Long-range PCR product
5. Microdissection or flow sorting
6. PRINS, In-situ PCR, Branched DNA labeling
7. *etc, etc, etc*
Non-radioactive Labelling techniques
Types of FISH probes

**LOCUS-SPECIFIC PROBE**

**ALPHOID OR CENTROMERIC REPEAT PROBE**

**CHROMOSOME-SPECIFIC PAINTING PROBE**

**METAPHASE**

**INTERPHASE NUCLEUS**
Locus specific probe

chromosome 13
sub-telomeric region probe
Whole chromosome paint probe

chromosome 4 probe
FISH Assay Design Strategies

- Enumeration
- Break-apart
- Single Fusion (with or without extra signal)
- Dual Fusion
Single Fusion Probes

nuc ish(ABL1,BCR)x2(ABL1 con BCRx1)[178/200]
CML

• Accounts for ~15-20% of all leukemia
• Most common in 40-50 year age group
• Neoplastic overproduction of granulocytes
• BM: granulocytic and megakaryocytic hyperplasia without maturation arrest
• Leukemogenic event at level of pluripotent stem cell
Cytogenetics of CML

- First neoplastic disease to be associated with a specific recurrent chromosome abnormality
- ~95% of patients exhibit a t(9;22)(q34;q11.2)
- der(22) is the Philadelphia chromosome
- simple variant translocations
- complex variant translocations
- Ph-negative CML
Classic t(9;22)(q34;q11.2) resulting in the BCR/ABL fusion in chronic myeloid leukemia
Single Fusion with Extra Signal

What is the ISCN nomenclature for each…?

Normal

3’ MBCR

5’ mBCR
1) What is the signal pattern?

2) What is the difference between a 5’ and 3’ break?

3) Is there a significance to the breakpoint?
Dual/Tri-colour Fusion Probes

nuc ish(ASS, ABL1, BCR)x2[200]

nuc ish(ASSx2, ABL1x3, BCRx3)(ASS con ABL1 con BCRx1)(ABL1 con BCRx1)[146/200]
For further clarification,
This is the tri-colour result.
Break-apart Probes

Normal – MLL Breakapart

MLL Rearrangement

nuc ish(5’MLL,3’MLL)x2 (5’MLL con 3’MLLx2)[200] (long-form)
Technical Hiccups

• Procedural Artifacts
  – Random chromosomal losses/gain
    • Culture artifacts
    • Mitotic errors
    • Nuclear truncation (paraffin sections)
  – Observational artifacts
    • 2D observation of a 3D structure
  – Specificity and sensitivity of hybridization
  – Others?
Clinical Applications
Prenatal Cytogenetics
Invasive Prenatal Testing

*Amniocentesis* (performed ~16 weeks of gestation)
• a fine needle is inserted through the abdominal wall and into the uterus. Approximately 15 ml of amniotic fluid is withdrawn from the surroundings of the developing fetus

*Chorionic Villus Sampling (CVS)* (performed ~10-12 weeks of gestation)
• cells from the developing placenta (the chorionic villi) are tested instead of amniotic fluid cells. The sample is obtained either by passing a small catheter through the vagina and cervix into the uterus, or by inserting a fine needle through the abdominal wall.
chorionic villi

amniotic fluid
Interphase FISH for prenatal specimens

The AneuVysion® Assay (CEP 18, X, Y-alpha satellite, LSI 13 and 21) Multi-color Probe Panel

- FISH probe set designed to detect most common aneuploidies

normal result

Consistent with Trisomy 21
Interphase FISH for prenatal specimens – Why?

Conventional cytogenetics
- large sample volume
- result in 2 weeks
- labour intensive

Interphase FISH
- small sample
- result in 2 days*
- less labour intensive

FISH assay does not detect the presence of structural chromosome abnormalities → routine chromosome analysis still required
Constitutional Cytogenetics
<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Chromosome</th>
<th>Start</th>
<th>End</th>
<th>Interval (Mb)</th>
<th>Grade</th>
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<tr>
<td>AZFB+AZFc</td>
<td>Y</td>
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<td>27,793,830</td>
<td>7.83</td>
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<td>16p11.2-p12.2 microdeletion syndrome</td>
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<td>21,613,956</td>
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<td>AZFB</td>
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<td>Prader-Willi syndrome (Type 1)</td>
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<td>22,876,632</td>
<td>28,557,186</td>
<td>5.68</td>
<td>1</td>
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<td>Angelman syndrome (Type 1)</td>
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<td>22,876,632</td>
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<td>65,071,919</td>
<td>68,645,525</td>
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<td>15q26 overgrowth syndrome</td>
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<td>99,357,970</td>
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<td>2q37 monosomy</td>
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<td>239,954,693</td>
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<td>AZFc</td>
<td>Y</td>
<td>24,977,425</td>
<td>28,033,929</td>
<td>3.06</td>
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<td>Miller-Diecker syndrome (MDS)</td>
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<td>175,130,402</td>
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<td>Sotos syndrome</td>
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<td>1</td>
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<tr>
<td>Williams-Beuren Syndrome (WBS)</td>
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<td>72,332,743</td>
<td>74,616,901</td>
<td>2.28</td>
<td>1</td>
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<td>7q11.23 duplication syndrome</td>
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<td>43,985,277</td>
<td>46,064,560</td>
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<tr>
<td>Potocki-Shaffer syndrome</td>
<td>11</td>
<td>43,985,277</td>
<td>46,064,560</td>
<td>2.08</td>
<td>1</td>
</tr>
</tbody>
</table>
Tupel 1 (22q11.2) Spectrum Orange DiGeorge Probe
ARSA (22q13.3) Spectrum Green Control Probe
del(22)(q11.2q11.2)
Cancer Cytogenetics
Cancer Applications

Chromosome analysis in cancer can be difficult due to several issues:

– Complex karyotypes (genomic instability)
– Heterogeneous tissue (mixed normal and tumour)
– Interpretation of meaningful results (diagnostic or predictive changes) from “innocent bystanders”
– Karyotyping tumours – chromosomes are often “short and ugly”
Cytogenetic Applications in Cancer

- Hematologic Malignancies
  - MDS
  - MPN
    - CML
  - NHL
    - Mantle Cell Lymphoma
    - Diffuse Large B-Cell Lymphoma
    - Follicular Lymphoma
    - Burkitt Lymphoma
    - Double-Hit Lymphoma
    - Anaplastic Large Cell Lymphoma
  - Plasma Cell Neoplasm
  - CLL

- Solid Tumours
  - Lipomatous Tumours
  - Fibromyxoid Chondrosarcoma
  - Clear Cell Carcinoma
  - Desmoplastic Small Round Cell Tumour
  - Synovial Sarcoma
  - Invasive Breast Cancer
  - Bladder Cancer
  - Ewing Sarcoma
  - PNET
  - Rhabdosarcoma
  - Neuroblastoma
  - Medulloblastoma
FICTION FISH

i.e. Fluorescence Immunophenotyping and Interphase Cytogenetics as a tool for the Investigation of Neoplasms

• This technique preserves cell morphology and combines immunofluorescence to detect cellular antigen and fluorescence in situ hybridization (FISH) to detect chromosomal abnormalities (Klaus Weber- Matthiesen et al 1992).

• Selection of cancer cells to analyze can improve sensitivity of genetic analysis
<table>
<thead>
<tr>
<th>Disorder</th>
<th>Disease definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGUS</td>
<td>Serum monoclonal protein level ≤3 g/dL, bone marrow plasma cells &lt;10%, and absence of end-organ damage, such as lytic bone lesions, anemia, hypercalcemia, or renal failure, that can be attributed to a plasma cell proliferative disorder</td>
</tr>
<tr>
<td>SMM (also referred to as asymptomatic multiple myeloma)</td>
<td>Serum monoclonal protein (IgG or IgA) level ≥3 g/dL and/or bone marrow plasma cells ≥10%, absence of end-organ damage, such as lytic bone lesions, anemia, hypercalcemia, or renal failure, that can be attributed to a plasma cell proliferative disorder</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>Bone marrow plasma cells ≥10%, presence of serum and/or urinary monoclonal protein (except in patients with true nonsecretory multiple myeloma), plus evidence of lytic bone lesions, anemia, hypercalcemia, or renal failure that can be attributed to the underlying plasma cell proliferative disorder</td>
</tr>
<tr>
<td>Solitary plasmacytoma</td>
<td>Biopsy-proven solitary lesion of bone or soft tissue with evidence of clonal plasma cells, normal skeletal survey, and MRI of spine and pelvis, and absence of end-organ damage, such as anemia, hypercalcemia, renal failure, or additional lytic bone lesions, that can be attributed to a plasma cell proliferative disorder</td>
</tr>
</tbody>
</table>

Ig = immunoglobulin; MGUS = monoclonal gammopathy of undetermined significance; MRI = magnetic resonance imaging; SMM = smoldering multiple myeloma.
Table 20-2  Myeloma chromosomal alterations.

Chromosome anomalies: incidence
  Conventional banding: 30%-50% of patients
  Interphase FISH: >90% of patients
  SKY: ~100%

Specific chromosome changes
  14q32: majority of cases
  11q13: most common (bcl-1 locus, 30%)
  4p16 (FGFR3, MMSET, 25%)
  8q24 (c-myc, 5%)
  16q23 (c-maf, 1%)
  6p25 (Irf4, rare)
  13 deletion (Rb)

Current Paradigm at BCCA

First-Line FISH

- t(4;14) FGFR3-IGH
- TP53/CEP17

Normal Signal Pattern for both probes

Analysis Complete

- t(4;14) –ve
- TP53 –ve
- IGH abnormal

Analysis Complete

- t(4;14) +ve OR
- TP53 +ve

- t(14;16) IGH-MAF
FICTION FISH images
44,X,-X,add(1)(p13),-5,add(6)(q15),add(7)(p15),add(8)(q24.1),+9,der(11)t(1;11)(p13;p11.2),
-13,der(13)t(1;13)(q12;p11),-14,add(14)(q32),-16,add(17)(p12),-20,+21,+21,+mar1

der(14)t(14;16)
der(16)t(14;16)
Soft Tissue Tumours

• Highly heterogeneous (~100 subtypes)
• Occur anywhere, but ¾ occur in the extremities
• Large majority are benign (>99%)
• Soft tissue sarcomas constitute less than 1% of all malignant tumours
• Differential diagnostic dilemmas are frequent and may include difficulty to distinguish benign from malignant
Cytogenetics of ALT/WDLS

- Supernumerary ring and giant marker chromosomes have been observed as the sole change or concomitant with a few other numerical or structural abnormalities.

- Supernumerary rings and giant markers are composed of interspersed amplified sequences consistently originating from the 12q14-15 region.

Genetic alterations in chromosome 12:
- 12q14.1 – CDK4
- 12q14.3 – HMGA2
- 12q15 – MDM2

Size: 11 MB
• Metaphase FISH with the presence of two giant supernumerary chromosomes. MDM2 probe stains both 12q15 and almost the entirety of both ESACs.

• This 12q14-15 amplification is not observed in lipomas and its detection may therefore serve to distinguish ALT/WD liposarcoma from benign adipose tumours.
Multicolour karyotyping - method

1. Prepare individual chromosome libraries
2. Label each chromosomal library using combination of different fluorochromes
3. Prepare a probe mixture of all 24 different labeled libraries + Cot-1 DNA
4. Denature the DNA
5. Hybridize to metaphase spreads
6. Acquire images
7. Classify the image

Adapted from The Principles of Clinical Cytogenetics 1999 Fig.11
Clinicopathological features of lymphoma/leukemia patients carrying both BCL2 and MYC translocations

Naoto Tomita,1 Mami Tokunaka,2 Naoya Nakamura,2 Kengo Takeuchi,3 Junki Koike,4 Shigeki Motomura,5 Ko Miyamoto,6 Ako Kikuchi,7 Rie Hyo,5 Yoshihiro Yakushijin,9 Yasufumi Masaki,50 Soichiro Fujii,11 Takamasa Hayashi,12 Yoshiaki Ishigatsubo,1 and Ikuo Miura13

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Table 3. Chromosomal analysis.

<table>
<thead>
<tr>
<th>UPN</th>
<th>Type</th>
<th>Abnormal karyotype</th>
<th>Cells</th>
<th>Sample</th>
<th>Ref</th>
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<tbody>
<tr>
<td>1</td>
<td>DHL-1</td>
<td>46,XY,add(1)(q21),add(1)(q32),add(3)(q21),add(6)(q21),t(8;14)(q24q32),t(14;18)(q32q21), 48, idem., +add(6), +10</td>
<td>6/20</td>
<td>BM</td>
<td>13</td>
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<td>2/20</td>
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<td>2/20</td>
<td>BM</td>
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<td>4</td>
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<td>19/20</td>
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<td>1/20</td>
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<td>4/16</td>
<td>BM</td>
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<td></td>
<td>12/16</td>
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Comparative Genomic Hybridization

- Predates chromosomal microarray
- Hybridize a target sample against a reference to look for gains and losses
- Resolution similar to that of low-resolution G-banding.
Chromosomal Microarray

**Array CGH: The Complete Process**

**Steps 1-3** Patient and control DNA are labeled with fluorescent dyes and applied to the microarray.

**Step 4** Patient and control DNA compete to attach, or hybridize, to the microarray.

**Step 5** The microarray scanner measures the fluorescent signals.

**Step 6** Computer software analyzes the data and generates a plot.
47,XX,add(10)(q11.2),der(19)t(1;19)(q12;p13),+add(21)(q22.3),2dmin[20]
Thank-you! Questions?
Cytogenetic Technologies (Overview)

• Traditional
  – G-banded Karyotyping

• Molecular Cytogenetic
  – Fluorescence in situ hybridization (FISH)
  – Multicolour Karyotyping mFISH/SKY

• Array Complete Genomic Hybridization
  – Targeted arrays/Whole Genome arrays
    • BAC / oligonucleotide / SNP-A

• Next Generation Sequencing
<table>
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<tr>
<th>Method</th>
<th>Blood</th>
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<th>Paraffin</th>
<th>Tumour Biopsy</th>
<th>Fine Needle Aspriate</th>
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<td>✓</td>
<td>✗</td>
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<td>✓</td>
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<td>✓</td>
</tr>
<tr>
<td>Array Complete Genomic Hybridization</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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</tr>
<tr>
<td>Next Generation Sequencing</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>
Mitotic Cell Cycle

- **G₁, G₂** = interphase
- **S** = replication
- **M** = mitosis

Stages:
- **Interphase**
  - **G₁**
  - **G₂**
- **Prophase**
- **Metaphase**
- **Anaphase**
- **Telophase**
<table>
<thead>
<tr>
<th>Technique</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-banded Karyotype</td>
<td>• Whole genome</td>
<td>• Cell of interest may not be dividing</td>
</tr>
<tr>
<td></td>
<td>• Interpretation of structural and numerical rearrangements relatively straightforward</td>
<td>• Highly labour intensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Highly trained technologist</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Resolution is between 5-10MB</td>
</tr>
<tr>
<td>FISH</td>
<td>• Highly sensitive for detection of rearrangements, amplification, deletion</td>
<td>• Individual or few loci</td>
</tr>
<tr>
<td></td>
<td>• Direct preparation</td>
<td>• Patterns can be complex to interpret</td>
</tr>
<tr>
<td></td>
<td>• Can subselect cell populations</td>
<td></td>
</tr>
<tr>
<td>mFISH/SKY</td>
<td>• Whole genome</td>
<td>• Expensive (not routine)</td>
</tr>
<tr>
<td></td>
<td>• Colours identify chromosomes automatically</td>
<td>• Requires metaphase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Breakpoints difficult to determine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• May miss cryptic rearrangements</td>
</tr>
<tr>
<td>Microarray</td>
<td>• Resolution (100-200X higher resolution than karyotype)</td>
<td>• Cannot detect balanced rearrangements or ploidy changes</td>
</tr>
<tr>
<td></td>
<td>• Automation</td>
<td>• No structure (molecular)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Interpretation?</td>
</tr>
<tr>
<td>Next Generation Sequencing</td>
<td>• Resolution (nucleotide level resolution)</td>
<td>• Cost (for now)</td>
</tr>
<tr>
<td></td>
<td>• Automation</td>
<td>• Too much information</td>
</tr>
<tr>
<td></td>
<td>• Can detect rearrangements (digital structure can be inferred)</td>
<td></td>
</tr>
</tbody>
</table>
Next Generation Sequencing

- **454**
  - Picolitre well
  - DNA polymerase and enzymes on beads
  - DNA immobilized on bead and amplified in water-oil emulsion
  - 250 nucleotides
  - 400,000 reads
  - 100 Mb

- **SOLiD**
  - Primer to adaptor
  - Random oligonucleotides with known 3' dinucleotide
  - Adaptor sequence
  - Coding scheme second base
  - First base
  - 35–50 nucleotides
  - 171 million reads
  - 6,000 Mb

- **Solexa GA**
  - Amplified DNA spots
  - Terminator dNTP
  - 50 nucleotides
  - 30 million reads
  - 1,500 Mb

- **Heliscope**
  - Single species dNTP
  - Unamplified immobilized DNA
  - 50 nucleotides
  - 30 million reads
  - 1,500 Mb

- **Pacific Biosciences**
  - Highly focused detection path
  - Detection volume
  - Immobilized DNA polymerase