Mutation Analysis in Frozen and FFPE Tumor Samples

Gad Getz, PhD
Kristin Ardlie, PhD

Broad Institute of Harvard and MIT
Why use FFPE?

- **Very** large numbers of samples in tissue banks and Biorepositories worldwide

- Samples often very well-characterized with histological, pathological and follow-up clinical data

- Can fill the accrual gap in TCGA (and future of TCGA)
  “We need to get to 10,000 patients per tumor type” -- Lou Staudt (Nov 2012)

- Remains part of clinical standard of care (difficult to change pathology practices for research needs alone)

- Enable connecting to existing clinical trials and move genomic analyses into standard clinical practice
Challenges with FFPE?

- Difficulty of extracting samples
  - Deparaffinization & de-cross-linking of protein-DNA.
  - Physical size of the samples can be small
  - Yield

- Poor quality of extracted material due to:
  - Warm-ischemic time in operating room
  - Type of formalin used, how fixed, & how long (un-buffered vs. buffered)
FFPE samples vary in size (TCGA samples)

FFPE Block Choices from Pilot Round #2

Group #1
(Large Tissues)

Group #2
(Medium Tissues)

Group #3
(Small Tissues)

Nationwide Children’s Hospital Biospecimen Core Resource
Samples from clinical study of drug resistance (Broad)
FFPE sample sets analyzed

- **TCGA Prostate** – “trios”
  - 4 FFPE Tumor samples + 4 Fresh Frozen Tumor/Normal pairs
  - Sequencing Coverage:
    - FFPE samples: 200x
    - Fresh Frozen pairs: 100x

- **Breast Cancer** – “trios”
  - 46 FFPE Tumor samples + 46 Fresh Frozen Tumor/Normal pairs
  - Source = FFPE Block, Mexico
  - Age of Fixed Block = 2008 – 2009 (plus a single 2010)

- **Lung Cancer, NSCLC Adenocarcinoma** – “quartets”
  - 17 FFPE Tumor/Normal sample pairs + 17 Fresh Frozen Tumor/Normal pairs
  - Source = FFPE Sections (15 microns, 9 per sample), Ontario, Canada
  - Age of Fixed Block = 2007 - 2010
Questions

1) Can we get high quality exome sequencing data from FFPE samples compared to frozen?

2) Can we detect mutations in FFPE samples? Are they artifacts?

3) Can we detect copy-number changes?

4) Are we finding the same mutations in FFPE vs. frozen?

5) Can we perform cancer genome projects using FFPE samples?

6) Can we use clinical FFPE samples for clinical decision making?
(1) Are we getting similar library sizes from whole-exome sequencing?

**% of Target Bases Covered**

- Frozen
- FFPE

**Estimated Library Size**

- Estimation of unique molecules in exome sequencing library

- % of target bases at 10x
- % of target bases at 20x
- % of target bases at 30x

*Carrie Sougnez*
(1) Are we getting similar coverage? TCGA Prostate Cancer

Coverage (\(\geq 14\ T/\geq 8\ N\)) is roughly the same across all samples in Frozen and FFPE ~30Mb of covered bases
(1) Similar results in 17 Lung quartets: coverage statistics
(2) Can we find mutations?
Total Count of mutations is similar

<table>
<thead>
<tr>
<th>Frozen</th>
<th>type</th>
<th>count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missense_Mutation</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>Nonsense_Mutation</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Silent</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Splice_Site</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Translation_Start_Site</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>135</td>
<td></td>
</tr>
</tbody>
</table>

Total territory: 130.49 MB

<table>
<thead>
<tr>
<th>FFPE</th>
<th>type</th>
<th>count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missense_Mutation</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>Nonsense_Mutation</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Nonstop_Mutation</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Silent</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Splice_Site</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Translation_Start_Site</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>137</td>
<td></td>
</tr>
</tbody>
</table>

Total territory: 130.66 MB

<table>
<thead>
<tr>
<th>Frozen</th>
<th>type</th>
<th>count</th>
</tr>
</thead>
<tbody>
<tr>
<td>De_novo_Start_OutOfFrame</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Frame_Shift_Del</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Frame_Shift_Ins</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>In_Frame_Del</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>In_Frame_Ins</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Missense_Mutation</td>
<td>3551</td>
<td></td>
</tr>
<tr>
<td>Nonsense_Mutation</td>
<td>236</td>
<td></td>
</tr>
<tr>
<td>Nonstop_Mutation</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Silent</td>
<td>1225</td>
<td></td>
</tr>
<tr>
<td>Splice_Site_DNF</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Splice_Site_SNP</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>Start_Codon_Del</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5332</td>
<td></td>
</tr>
</tbody>
</table>

Total territory: 499.2 Mb

<table>
<thead>
<tr>
<th>FFPE</th>
<th>type</th>
<th>count</th>
</tr>
</thead>
<tbody>
<tr>
<td>De_novo_Start_OutOfFrame</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Frame_Shift_Del</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Frame_Shift_Ins</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>In_Frame_Del</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>In_Frame_Ins</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Missense_Mutation</td>
<td>3428</td>
<td></td>
</tr>
<tr>
<td>Nonsense_Mutation</td>
<td>270</td>
<td></td>
</tr>
<tr>
<td>Nonstop_Mutation</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Silent</td>
<td>1152</td>
<td></td>
</tr>
<tr>
<td>Splice_Site_DNF</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Splice_Site_SNP</td>
<td>122</td>
<td></td>
</tr>
<tr>
<td>Splice_Site_TNP</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5013</td>
<td></td>
</tr>
</tbody>
</table>

Total territory: 512.2 Mb
(2) Are the FFPE mutations swamped by artifacts? No! The mutations have the same spectra.
(3) Can we detect copy number changes? Example 1

Using CapSeg, Aaron McKenna, Scott Carter
(3) Can we detect copy number changes? Example 2

Using CapSeg, Aaron McKenna, Scott Carter
(4) Are we finding the same mutations in FFPE and frozen?
Overlap between FFPE and Frozen (17 lung)

a. Venn diagram showing the overlap between FFPE and Frozen samples.

b. Bar graph showing relative mutation rates for different mutation categories.

c. Scatter plot comparing FFPE and Frozen allele fractions.

d. Curve showing the percentage of overlap against minimum allele fraction.
Overlap between FFPE and Frozen samples (4 prostate)

![Venn diagram showing overlap between FFPE and Frozen samples. The diagram indicates that 56 out of 77 FFPE samples overlap with 79 Frozen samples, with an overlap percentage of 26.5%.]

Scatter plot for individual set:

- Green dots: FFPE and Frozen
- Blue dots: FFPE only
- Red dots: Frozen only

![Scatter plot showing allele fraction comparison between FFPE and Frozen samples. The plot includes error bars for each data point.]

![Graph showing % overlap against min allele fraction. The graph includes error bars for each data point, indicating variability in the overlap percentage.]
A fundamental observation: When comparing frozen to FFPE we are changing TWO variables at once

(1) Frozen vs FFPE
(2) Two different pieces of the tumor
   -- Different in terms of tumor purity
   -- Different with respect to sub-clonal composition

THIS AFFECTS ALL COMPARISONS BETWEEN FFPE AND FROZEN SAMPLES (DNA, RNA, PROTEINS)
Sensitivity to detect (and even observe) a mutation – depends on coverage and allelic fraction

The ability to detect mutations depends on the **coverage** and **mutation allelic fraction** (the expected fraction of reads that support a mutation)

Purity = 67%
Absolute copy number in tumor = 4
Mutation multiplicity = 1
⇒ Allelic fraction = 2/10 = 0.2


**ABSOLUTE**: SNP arrays / exome sequencing ⇒ purity, ploidy & abs. copy-number profile
Allelic fraction in frozen and FFPE are different due to differences in purities (17 lung)
ABSOLUTE can distinguish between clonal & sub-clonal mutations

Ovarian cancer

~50% are subclonal mutations

How should we compare the FFPE and frozen mutation sets?

1) We do not need to independently call the mutation in both FFPE and frozen. All we need is to validate the existence of the mutations found in FFPE in the frozen sample (i.e. call with a lower stringency since testing only a small number of mutation) \(\Rightarrow\) require 2+ reads

2) Correct for the different allelic fraction in the two samples due to different purity of FFPE and frozen \(\Rightarrow\) fit a line

3) Stratify sites based on the power to validate a mutation \(\Rightarrow\) 80%, 95%

4) Distinguish between clonal and sub-clonal mutations \(\Rightarrow\) use ABSOLUTE to assign mutation as clonal or sub-clonal
Minimum number of reads to have power of 80%

Minimum no. of reads

Allelic frac0on of mutation

60 reads

135 reads

2+ 3+ 4+ 5+
Minimum number of reads to have power of 95%

- Minimum number of reads to have power of 95% is 93 reads for 2+ alleles.
- Minimum number of reads to have power of 95% is 180 reads for 3+ alleles.
Validate = 2+ reads, AF corrected, power of 80%

**All**

Number of validated, invalidated and unpowered sites per sample

**Clonal (based on ABSOLUTE on FFPE)**

Number of validated, invalidated and unpowered sites per sample

- **Invalidated**
- **Validated**
- **Validated + called**
- **Unpowered**
Validate = 2+ reads, AF corrected, power of 95%

All

Clonal (based on ABSOLUTE on FFPE)
Validate = 2+ reads, AF corrected, power of 80% and 95%

**All**

Percentage of sites powered, called, and validated per sample.

**Clonal (based on ABSOLUTE on FFPE)**

Percentage of sites powered, called, and validated per sample.

- % powered
- validated
- Validated + called

80%

95%
Can we perform cancer genome projects using FFPE samples?

Yes! Very similar MutSig lists

Orange background if within top 30 of other list

MutSig: Significant genes (17 samples)

<table>
<thead>
<tr>
<th>Frozen</th>
<th>FFPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Table of significant genes]</td>
<td>![Table of significant genes]</td>
</tr>
</tbody>
</table>

Old MutSig version
(6) Can we sequence clinical FFPE samples for clinical decision making? Yes!

<table>
<thead>
<tr>
<th>Gene</th>
<th># in Frozen</th>
<th># in FFPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>KRAS</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>EGFR</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>STK11</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>KEAP1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>ATM</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>NF1</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

17 lung samples
Conclusions

• Exome Sequencing of FFPE samples is robust – we can extract DNA, capture and sequence
• We can calculate overlap between FFPE and frozen samples controlling for relative coverage and adjust for different allelic fractions
• Mutation rates and categories are very similar
• Sub-clonal mutations contribute to the differences

➡️ We can perform cancer genome project based on FFPE material
➡️ We can use clinical FFPE samples for exome sequencing

• We are still analyzing more data in order to get reach final conclusions
Ongoing challenges

- WGS requires samples to be larger size range than exome (and sample prep more sensitive to changes that formalin fixation causes on DNA)
  - may not be suitable for samples that are highly degraded.
  - May need to optimize extraction steps to de-crosslink samples

- Low yield samples – small valuable specimens or micro-dissected samples

- Older blocks – may be very valuable but more variable due to storage conditions and older practices, such as use of unbuffered formalin (causes more DNA/RNA sample damage and cross-linking).
Acknowledgements

NCI/NHGRI

Kristin Ardlie
Petar Stojanov
Andrey Sivachenko
Scott Carter
Mike Lawrence
Carrie Sougnez
Daniel Auclair
Marcin Imilienski
Kristian Cibulskis

Stacey Gabriel
Matthew Meyerson
Todd Golub
Eric Lander

Broad
Biological Sample Platform
Genetic Analysis Platform
Sequencing Platform

TCGA
Kenna Shaw
Brad Ozenberger
NCH BCR
THE END