High Fidelity Copy Number Analysis of Formalin-Fixed and Paraffin-Embedded Tissues Using Affymetrix Cytoscan HD Chip

Yan P. Yu¹, Amantha Michalopoulos¹, Ying Ding², George Tseng², Jian-Hua Luo¹*¹

¹ Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States of America, ²Department of Statistics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States of America

Abstract
Detection of human genome copy number variation (CNV) is one of the most important analyses in diagnosing human malignancies. Genome CNV detection in formalin-fixed and paraffin-embedded (FFPE) tissues remains challenging due to suboptimal DNA quality and failure to use appropriate baseline controls for such tissues. Here, we report a modified method in analyzing CNV in FFPE tissues using microarray with Affymetrix Cytoscan HD chips. Gel purification was applied to select DNA with good quality and data of fresh frozen and FFPE tissues from healthy individuals were included as baseline controls in our data analysis. Our analysis showed a 91% overlap between CNV detection by microarray with FFPE tissues and chromosomal abnormality detection by karyotyping with fresh tissues on 8 cases of lymphoma samples. The CNV overlap between matched frozen and FFPE tissues reached 93.8%. When the analyses were restricted to regions containing genes, 87.1% concordance between FFPE and fresh frozen tissues was found. The analysis was further validated by Fluorescence In Situ Hybridization on these samples using probes specific for BRAF and CITED2. The results suggested that the modified method using Affymetrix Cytoscan HD chip gave rise to a significant improvement over most of the previous methods in terms of accuracy in detecting CNV in FFPE tissues. This FFPE microarray methodology may hold promise for broad application of CNV analysis on clinical samples.

Introduction
Genome abnormalities are the hallmark of human malignancies [1]. These include chromosome deletion, amplification, translocation, inversions and isochromosome formation. Analysis of genome abnormality is critical in making diagnosis of human malignancies, congenital birth defects and a variety of inheritable diseases. Array comparative genome hybridization (aCGH) or Affymetrix SNP array has been frequently applied to clinical samples to examine loss of heterozygosity and to detect amplification or deletion of genome fragments in the chromosomes [2–8]. The current methodologies using aCGH or Affymetrix SNP6.0 require high quality genome DNA from fresh frozen tissues. However, most of the samples for pathological evaluation are formalin-fixed and paraffin-embedded (FFPE) tissue blocks. Suboptimal and high background results are obtained when tissues from FFPE tissue blocks are analyzed due to the fragmenting nature of genome DNA in FFPE tissues. The low quality of genome copy number analysis from FFPE tissues practically precludes the application of whole genome copy number variation (CNV) analyses in clinical setting. Thus, a new method that can reproducibly generate high quality CNV analysis from FFPE tissues is needed to make high throughput genome CNV analysis applicable to clinical setting.

Lymphoma is one of the human malignancies that are frequently associated with large number of structural genome abnormalities. The classification and treatment of lymphomas are based on their genotypes in the tumor cells. Thus, lymphoma is an ideal human malignancy to investigate whether FFPE tissue is suitable for CNV analysis using Affymetrix Cytoscan HD chip. In this report, we describe a method that is adapted to the genomic DNA extracted from FFPE tissues to prepare the DNA cocktail for Affymetrix Cytoscan HD analysis. To validate this method, frozen genome DNA samples from matched lymphoma cases were also analyzed on Affymetrix Cytoscan HD chips. The results showed close overlaps in CNV profiles between FFPE and matched frozen tissues.

Materials and Methods
Tissue samples
Fresh frozen tissues of eight cases of human malignant lymphomas, including 5 diffused large B cell lymphomas, 2 follicular lymphomas and 1 T cell non-Hodgkins lymphoma, were obtained from clinical services. These tissues were dissected to have at least 70% purity of tumor cells. The study was approved by University of Pittsburgh Medical Center Quality Insurance Committee and Institutional Review Board, and exempted from
| Case  | Frozen genotype | FFPE genotype | Karyotype |
|-------|----------------|---------------|-----------|
|       | Chr | Locus | Copy | Def | Size | Locus | Copy | Def | Size |          |
|       | 2   | 2p16.1 | 1    | Loss | 0.7 MB | 2p16.1 | 1    | Loss | 0.5 MB | –2 |
|       | 5   | 5q13.2-14.3 | 1.3 | Loss | 18.4 MB | 5q13.2-14.3 | 1.4 | Loss | 17 MB | –5 |
|       | 7   | 7p14.3-14.1 | 1.3 | Loss | 6.5 MB | 7p14.3-14.1 | 1.5 | Loss | 6.7 MB | der(7)(7;?)q11.2?) |
|       | 8   | 8p23.3-11.2 | 1.2 | Loss | 42.6 MB | 8p23.3-11.2 | 1.4 | Loss | 39.6 MB | –8 [17] |
|       | 9   | 9p24.1 | 4    | Gain | 2.0 MB | 9p24.1 | 4    | Gain | 2.0 MB |          |
|       | 10  | 10p15.3-15.1 | 1.5 | Loss | 10.1 MB | 10p15.4-14.3 | 1.5 | Loss | 14.2 MB |          |
|       | 14  | 14q23.3-24.3 | 1.2 | Loss | 2.8 MB | 14q23.3-24.3 | 1.2 | Loss | 2.8 MB |          |
|       | 17  | 17p13.3-13.1 | 2.5 | Gain | 8.5 MB | 17p13.3-13.1 | 2.5 | Gain | 8.7 MB | –17 |
|       | 18  | 18p11.2-11.1 | 1.5 | Loss | 33 MB | 18p11.2-11.1 | 1.5 | Loss | 33 MB | –16 |
|       | 19  | 19p12.1-12.2 | 1.5 | Gain | 15.5 MB | 19p12.1-12.2 | 1.5 | Gain | 15.5 MB |          |
|       | 21  | 21q22.1-22.2 | 2.5 | Gain | 2.7 MB | 21q22.1-22.2 | 2.5 | Gain | 2.7 MB |          |
|       | 22  | 22q11.2 | 1    | Loss | 0.9 MB | 22q11.2 | 1    | Loss | 1.0 MB |          |
| Case 3 | 1  | Xp22.3-23.2  | 3    | Gain | 156 MB | Xp22.3-23.2  | 3    | Gain | 156 MB | +X |
|       | 5   | 5p15.33-35.3 | 2.6 | Gain | 180.6 MB | 5p15.33-35.3 | 2.6 | Gain | 180.6 MB | 5 |
|       | 8   | 8p23.3-21.3 | 1.4 | Loss | 22.8 MB | 8p23.3-21.3 | 1.4 | Loss | 22.3 MB | del(8)(p21p23) |
|       | 9   | 9q24.1-24.3 | 2.5 | Gain | 4.7 MB | 9q24.1-24.3 | 2.5 | Gain | 7.1 MB | add(9)(q22) |
|       | 10  | 10p15.3 | 1.4 | Gain | 0.8 MB | 10p15.3 | 1.5 | Loss | 0.8 MB | dic(1;10)(10qter>10p15:1p13>1q25:1q21>1q32:1q25>1qter) |
|       | 11  | 11p14.2-11.2 | 2.5 | Gain | 23.6 MB | 11p14.2-11.2 | 2.4 | Gain | 23.6 MB |          |
|       | 13  | 13q33.3-34 | 1.3 | Loss | 6.6 MB | 13q33.3-34 | 1.3 | Loss | 6.7 MB | add(13)(q34) |
|       | 18  | 18p11.2-11.1 | 1.5 | Loss | 33 MB | 18p11.2-11.1 | 1.5 | Loss | 33 MB | –16 |
|       | 21  | 21q22.1-22.2 | 2.5 | Gain | 2.7 MB | 21q22.1-22.2 | 2.5 | Gain | 2.7 MB |          |
|       | 22  | 22q11.2 | 1    | Loss | 0.9 MB | 22q11.2 | 1    | Loss | 1.0 MB |          |

Table 1. Genotyping concordance between FFPE and Frozen tissues.
| Case | Frozen genotype | FFPE genotype | Karyotype |
|------|----------------|---------------|-----------|
|      | Chr | Locus | Copy | Def | Size | Locus | Copy | Def | Size |          |
| 18   | 18  | p11.32-q11.2 | 3    | Gain | 22 MB | 18p11.32-q11.2 | 3    | Gain | 22 MB | 18      |
| 18   | 18  | q11.2-23 | 4    | Gain | 55 MB | 18q11.2-23 | 4    | Gain | 55 MB | 18      |
| 19   | 19  | p13.2-13.43 | 2.7  | Gain | 18.1 MB | 19p13.2-13.43 | 2.6  | Gain | 19.4 MB | 19      |
| X    | X   | p22.33-q28 | 3    | Gain | 156 MB | Xp22.33-q28 | 3    | Gain | 156 MB | +X      |
| Case 4 | 1   | p13.1   | 1.2  | Loss | 13.7 MB | 1p31.1 | 1.4  | Loss | 13.2 MB | 78-79,inc[cp5]* |
|       | 1q23.3-32.1 | 2.3  | Gain | 31.4 MB | 1q23.3-32.1 | 2.3  | Gain | 29.5 MB |         |
| 2    | 2   | p22.4-44 | 1.2  | Loss | 17.2 MB | 2q22.4-44 | 1.5  | Loss | 10 MB |          |
|       | 2p25.3-24.3 | 2.7  | Gain | 33 MB | 2p25.3-24.3 | 2.3  | Gain | 14.3 MB |          |
| 3    | 3   | p24.3-16.3 | 1.5  | Loss | 34 MB | 3p24.3-16.3 | 1.6  | Loss | 34.1 MB |          |
| 4    | 4   | p16.3-24.1 | 1.6  | Loss | 106.6 MB | 4p16.3-24.1 | 1.6  | Loss | 104.6 MB |          |
|       | 4q25-34.1 | 2.7  | Gain | 64.7 MB | 4q25-34.1 | 2.5  | Gain | 65.5 MB |          |
| 5    | 5   | p15.33-15.31 | 2.6  | Gain | 18.1 MB | 5p15.33-15.31 | 3    | Gain | 0.7 MB |          |
| 7    | 7   | p22.3-21.1 | 1.1  | Loss | 17.8 MB | 7p22.3-21.1 | 1.4  | Loss | 17.3 MB |          |
|       | 7q11.22-36.3 | 3    | Gain | 93.5 MB | 7q11.22-36.3 | 2.8  | Gain | 93.6 MB |          |
| 8    | 8   | p23.3-12 | 1.6  | Loss | 32.9 MB | 8p23.3-12 | 1.6  | Loss | 30.9 MB |          |
| 9    | 9   | p23.1-22.3 | 0.8  | Loss | 8.5 MB | 9p23.1-22.3 | 1.2  | Loss | 8.1 MB |          |
| 10   | 10  | p21.3-21.1 | 0.7  | Loss | 7 MB | 10p21.3-21.1 | 1    | Loss | 6.9 MB |          |
|       | 9p21.1 | 0.8  | Loss | 1 MB | 9p21.1 | 1.3  | Loss | 1 MB |          |
| 11   | 11  | p13.3 | 0.9  | Loss | 13 MB | 11p13.3 | 1.3  | Loss | 13 MB |          |
|       | 9q22.3-22.31 | 0.7  | Loss | 2.5 MB | 9q22.2-22.31 | 1    | Loss | 2.4 MB |          |
| 12   | 12  | p22.31-22.33 | 1.2  | Loss | 4.4 MB | 9q22.31-22.33 | ND | NA | NA |          |
| 13   | 13  | q31.1-31.2 | 0.8  | Loss | 6.3 MB | 9q31.1-31.2 | 1.1  | Loss | 5.6 MB |          |
|       | 9q32-34.11 | 1    | Loss | 15.1 MB | 9q32-34.11 | 1.3  | Loss | 17 MB |          |
| 14   | 14  | p11.2-21.1 | 0.3  | Loss | 0.5 MB | 14p11.2 | 0.7  | Loss | 0.6 MB |          |
|       | 14q32.32 | 0.7  | Loss | 0.6 MB | 14q32.32 | 1.2  | Loss | 0.6 MB |          |
| 15   | 15  | p13.3-13.2 | 1.2  | Loss | 5.9 MB | 15p13.3-13.2 | 1.5  | Loss | 5.8 MB |          |
| 16   | 16  | q21.3-13.1 | 0.8  | Loss | 1.7 MB | 16q21.3-13.1 | 1.3  | Loss | 0.9 MB |          |
| 17   | 17  | p13.1-11.2 | 1.2  | Loss | 14.4 MB | 17p13.1-11.2 | 1.3  | Loss | 13.4 MB |          |
| 18   | 18  | q11.1-25.3 | 3.2  | Gain | 55.7 MB | 18q11.1-25.3 | 2.9  | Gain | 54.2 MB |          |
| 19   | 19  | p13.2-11.2 | 3.2  | Gain | 30.4 MB | 19p13.2-11.2 | 2.7  | Gain | 31.1 MB |          |
| 20   | 20  | p12.2-22.3 | 1.5  | Loss | 33.1 MB | 20p12.2-22.3 | 1.7  | Loss | 23 MB |          |
| 21   | 21  | p12.2-22.3 | 1.5  | Loss | 27.4 MB | 1p11.2-q21.2 | 2.5  | Gain | 33.3 MB |          |
### Table 1. Cont.

| Case | Frozen genotype | FFPE genotype | Karyotype |
|------|-----------------|---------------|-----------|
|      | Chr Locus Copy Def Size | Locus Copy Def Size |                |
| 4    | 4p16.3-q36.2 1.4 Loss 190.9 MB | 4p16.3-q36.2 1.6 Loss 188.2 MB | –4          |
| 5    | 5p15.33-q11.2 3.2 Gain 58.8 MB | 5p15.33-q11.2 2.5 Gain 57.5 MB | +5, i(5)(p10) |
| 6    | 6p25.3-21.1 3.1 Gain 45 MB | 6p25.3-21.1 2.5 Gain 45.3 MB |           |
| 7    | 7p22.3-14.1 2.7 Gain 41.9 MB | 7p22.3-14.1 2.5 Gain 45.2 MB |           |
| 13   | 13q31.1-34 3 Gain 35.8 MB | 13q31.1-34 2.5 Gain 35.8 MB | add(13)(q34) |
| 18   | 18q21.11-21.33 3.2 Gain 28.4 MB | 18q21.11-21.33 2.7 Gain 28.2 MB | add18(q21) |
| 20   | 20p13-q11 2.6 Gain 38.1 MB | 20p13-q11 2.5 Gain 38.1 MB |           |
|      | 1p12-q44 2.4 Gain 128.8 MB | 1p12-q44 2.4 Gain 128.2 MB | +1,add(1)(p32) |
| 2    | 2p25.3-16.1 1.6 Loss 58.6 MB | 2p25.3-16.1 1.6 Loss 58.1 MB | –2          |
| 3    | 3p14.1-13 3.9 Gain 7.6 MB | 3p14.1-13 3.5 Gain 7.6 MB | 3           |
| 4    | 4p16.3-q35.2 1.6 Loss 190.9 MB | 4p16.3-q35.2 1.6 Loss 190.9 MB | –4          |
| 5    | 5p15.33-q11.2 3.2 Gain 58.8 MB | 5p15.33-q11.2 2.5 Gain 57.5 MB | +5, i(5)(p10) |
| 6    | 6p26.3-11.2 2.4 Gain 58.3 MB | 6p26.3-11.2 2.5 Gain 57.3 MB | +6,(6)(p10) |
| 7    | 7p22.3-36.3 2.4 Gain 159 MB | 7p22.3-36.3 2.4 Gain 159 MB | 7           |
| 10   | 10p16.3-15.2 1.6 Loss 3.6 MB | 10p16.3-15.2 1.7 Loss 1.8 MB | dic(10)(q10)p13 |
| 17   | 17p13.3-q11.1 1.6 Gain 25.6 MB | 17p13.3-q11.1 1.6 Gain 25.9 MB | i(17)(q10) |
| 20   | 20p12.3-12.1 1.5 Loss 5.9 MB | 20p12.3-12.1 1.6 Loss 5.9 MB | –20          |
| 11   | 11q12.1-13.5 1.6 Loss 96.7 MB | 11q12.1-13.5 1.7 Loss 96.7 MB | i(1)(q10) |
| 2    | 2p25.3-25.2 1.7 Loss 6.3 MB | 2p25.3-25.2 1.7 Loss 5.0 MB |           |
|      | 2p21-13.3 2.4 Gain 22.1 MB | 2p21-13.3 2.4 Gain 17.2 MB | add(2)(p11.2) |
| 3    | 3p26.3-12.1 1.7 Loss 61.1 MB | 3p26.3-12.1 1.7 Loss 61.1 MB | del(3)(p13p25) |
| 4    | 4p16.3-q35.2 1.6 Loss 188.5 MB | 4p16.3-q35.2 1.7 Loss 188.5 MB | –4          |
| 5    | 5p15.33-q11.2 3.2 Gain 58.8 MB | 5p15.33-q11.2 2.5 Gain 57.5 MB | +5, i(5)(p10) |
| 6    | 6p26.3-11.2 2.4 Gain 58.3 MB | 6p26.3-11.2 2.5 Gain 57.3 MB | +6,(6)(p10) |
| 7    | 7p22.3-36.3 2.4 Gain 159 MB | 7p22.3-36.3 2.4 Gain 159 MB | 7           |
| 10   | 10p16.3-15.2 1.6 Loss 3.6 MB | 10p16.3-15.2 1.7 Loss 1.8 MB | dic(10)(q10)p13 |
| 17   | 17p13.3-q11.1 1.6 Gain 25.6 MB | 17p13.3-q11.1 1.6 Gain 25.9 MB | i(17)(q10) |
| 20   | 20p12.3-12.1 1.5 Loss 5.9 MB | 20p12.3-12.1 1.6 Loss 5.9 MB | –20          |
| 20   | 20q11.1-13.3 1.6 Loss 33.3 MB | 20q11.1-13.3 1.6 Loss 33.1 MB |           |
|      | 1p12-q44 2.4 Gain 128.8 MB | 1p12-q44 2.4 Gain 128.2 MB | +1,add(1)(p32) |
| 2    | 2p25.3-16.1 1.6 Loss 58.6 MB | 2p25.3-16.1 1.6 Loss 58.1 MB | –2          |
| 3    | 3p14.1-13 3.9 Gain 7.6 MB | 3p14.1-13 3.5 Gain 7.6 MB | 3           |
| 4    | 4p16.3-q35.2 1.6 Loss 190.9 MB | 4p16.3-q35.2 1.6 Loss 190.9 MB | –4          |
| 5    | 5p15.33-q11.2 3.2 Gain 58.8 MB | 5p15.33-q11.2 2.5 Gain 57.5 MB | +5, i(5)(p10) |
| 6    | 6p26.3-11.2 2.4 Gain 58.3 MB | 6p26.3-11.2 2.5 Gain 57.3 MB | +6,(6)(p10) |
| 7    | 7p22.3-36.3 2.4 Gain 159 MB | 7p22.3-36.3 2.4 Gain 159 MB | 7           |
| 10   | 10p16.3-15.2 1.6 Loss 3.6 MB | 10p16.3-15.2 1.7 Loss 1.8 MB | dic(10)(q10)p13 |
| 17   | 17p13.3-q11.1 1.6 Gain 25.6 MB | 17p13.3-q11.1 1.6 Gain 25.9 MB | i(17)(q10) |
| 20   | 20p12.3-12.1 1.5 Loss 5.9 MB | 20p12.3-12.1 1.6 Loss 5.9 MB | –20          |
| 20   | 20q11.1-13.3 1.6 Loss 33.3 MB | 20q11.1-13.3 1.6 Loss 33.1 MB |           |

NP-normal ploidy; ND-Not detected; NA-Not applicable; Def-copy number variation definition; *-No analysis was reported due to low number of cells survived.

doi:10.1371/journal.pone.0092820.t001
informed-consent. The matched FFPE tissues from the same patients were also frozen sectioned onto slides, fixed and dehydrated with 100% ethanol and similarly micro-dissected to obtain tumor cells. Karyotyping analyses were performed on all these cases to detect chromosome abnormalities.

**Affymetrix CytoScan HD chip analysis of copy number variation of tumor cells**

For macrodissected frozen tissues, DNA was extracted using QIAamp blood and tissue kit (Qiagen, Valencia, CA). Five hundred nanograms of genome DNA were digested with Nsp1 for 2 hours at 37°C. The digested DNA was purified and ligated with primer/adaptors at 16°C for 12–16 hours. Amplicons were generated by performing PCR using primers provided by the manufacturer (Affymetrix, CA) on the ligation products using the following program: 94°C for 3 min, then 35 cycles of 94°C 30 second, 60°C for 45 sec and 65°C for 1 minute. This was followed by extension at 68°C for 7 min. The PCR products were then purified and digested with DNaseI for 35 min at 37°C to fragment the amplified DNA. The fragmented DNA was then labeled with biotinylated nucleotides through terminal deoxynucleotide transferase for 4 hours at 37°C. Two hundred fifty micrograms of fragmented DNA were hybridized with a pre-equilibrated Affymetrix chip Cytoscan HD chip at 50°C for 18 hours. The procedures of washing and scanning of CytoscanHD chips followed the manuals provided by Affymetrix, Inc. Cel files were generated from AGCC software from Affymetrix, Inc. (Santa Clara, CA). For FFPE tissues, micro-dissected tumor cells were treated with xylene for 12 hours. DNA was then extracted using QiaAmp FFPE DNA extraction kit. Gel purification of DNA sizes ranging from 200 to 1000 bp was performed. Two hundred fifty nanograms of purified DNA was then digested with NSP1, and similarly processed as frozen tissues.

**Statistical analysis**

Sixteen cel files were analyzed with Genotyping console for quality control analysis. Samples with QC call above 80% were admitted into the analysis. To analyze CNV, cel files were imported into Chromosome Analysis Suite 1.2 (Affymetrix, Inc) to generate copy number from raw intensity. For frozen tissues, Cytoscan HD files from fresh frozen tissues of 380 healthy individuals provided by Affymetrix were used as a baseline. For FFPE tissues, Cytoscan HD files from FFPE tissue of 100 healthy individuals from Affymetrix were used. Deletions or amplifications of genomes were analyzed by first limiting to the regions with p-value less than 0.05/total number of regions detected, i.e. family-wise error rate (FWER) is controlled using Bonferroni’s correction [9]. The selected regions were filtered by limiting to the regions with at least 25 markers and 500 kb. For genome fragment gain determination, a mean of >2.3 for autosomal chromosomes or
83000 filter set with single band exitors for Texas Red/Quips Genetic Workstation equipped with Chroma Technology DAPI. Analysis was performed using a Nikon Optiphot-2 and Slides were then air-dried in the dark and counterstained with 20 min. Slides were washed 3 times for 2 min each in fresh PBS. slipping and incubating in a moist chamber in the dark at 37

Avidin-spectrum orange (Zymed, San Francisco, CA), cover-

for 2 min. The biotin label was visualized by conjugation with phosphate buffered saline (PBS) at room temperature in the dark

cement, placed in a humidified chamber and hybridized overnight

probe was placed on the slide, cover-slipped, sealed with rubber

formamide for 3 min, and dehydrated in 70%, 85%, and 100%

CITED2 or BRAF (150 Kb)/50% formamide with 1

direct-

u

C. Coverslips were removed and the slides were washed in

6

10

2.2

16; Pearson correlation coefficient = 0.72.

Table 2. CNV Overlaps between Frozen tissues and matched formalin-fixed paraffin-embedded tissues.

| Case | Loss by Frozen | loss by FFPE | Overlap | Gain by Frozen | Gain by FFPE | Overlap |
|------|----------------|--------------|---------|----------------|--------------|---------|
| Case 1 | 170.4 MB | 170.3 MB | 91.70% | 180.8 MB | 177.3 MB* | 92.70% |
| Case 2 | 93.4 MB | 97.1 MB | 95.60% | 311.3 MB | 311.3 MB | 99.60% |
| Case 3 | 24.3 MB | 24.1 MB | 98.80% | 523.1 MB | 523.1 MB | 98.60% |
| Case 4 | 614.9 MB | 545 MB | 82.10% | 334.9 MB | 327.5 MB | 94.90% |
| Case 5 | 50.1 MB | 49.7 MB | 96.80% | N/D | N/D | N/A |
| Case 6 | 329.4 MB | 302.8 MB | 91.90% | 275.4 MB | 283.4 MB | 95.90% |
| Case 7 | 326.2 MB | 321.7 MB | 98.40% | 362.7 MB | 360.8 MB | 99.60% |
| Case 8 | 829.4 MB | 734.9 MB | 88.70% | 468.1 MB | 527.9 MB | 82% |

N/D-Not detected; N/A-Not applicable.

Table 3. Correlation of CNV callings between FFPE and matched fresh frozen tissues by segment number.

| CNV | fresh gain | fresh loss |
|-----|------------|-----------|
| FFPE loss | 68 | 18 |
| FFPE gain | 13 | 352 |

P<2.2×10−6; Pearson correlation coefficient = 0.72. doi:10.1371/journal.pone.0092820.t003

>1.5 for sex chromosomes of male was required, while for genome fragment loss determination, a mean of <1.7 for autosomal chromosomes or <0.5 of sex chromosomes of male was required. Loss of heterozygosity was not analyzed due to lack of matched normal tissues.

Fluorescence In-situ Hybridization (FISH)

Tissue slides (5 microns) were placed in 2×SSC at 37°C for 30 min. Slides were then removed and dehydrated in 70% and 85% ethanol for 2 min each at room temperature, and air dried. The probes for CITED2 and BRAF FISH analysis were obtained from BACPAC Resource Center, Oakland, CA. The DNA from the selected clone was extracted using Nucleobond Ax kit (Macherey-Nagel, Easton, PA). The probe was prepared by combining 7 μl of biotin-labeled genomic sequence containing CITED2 or BRAF (150 Kb)/50% formamide with 1 μl of direct-labeled CEP7 spectrum green for BRAF or CEP6 for CITED2 (Vysis, Downers Grove, IL). The probe was denatured for 5 min at 75°C. Sections of formalin-fixed tissues were denatured in 70% formamide for 3 min, and dehydrated in 70%, 85%, and 100% ethanol for 2 min each at room temperature. The denatured probe was placed on the slide, cover-slipped, sealed with rubber cement, placed in a humidified chamber and hybridized overnight at 37°C. Coverslips were removed and the slides were washed in 2×SSC/0.3% NP-40 at 72°C for 2 min. Slides were then held in phosphate buffered saline (PBS) at room temperature in the dark for 2 min. The biotin label was visualized by conjugation with Avidin-spectrum orange (Zymed, San Francisco, CA), cover-slipping and incubating in a moist chamber in the dark at 37°C for 20 min. Slides were washed 3 times for 2 min each in fresh PBS. Slides were then air-dried in the dark and counterstained with DAPI. Analysis was performed using a Nikon Optiphot-2 and Quips Genetic Workstation equipped with Chroma Technology 83000 filter set with single band exitors for Texas Red/Rhodamine, FITC and DAPI (uv 360 nm). Only individual and well delineated cells with two hybridization signals were scored. Overlapping cells were excluded from the analysis. Fifty to 100 cells per sample were scored to obtain an average of signals.

Karyotyping analysis

Cytogenetic analysis was performed on cell cultures of lymphoma samples. The cell cultures were stimulated with phytohemagglutinin (PHA) for 72 hours, and harvested and treated briefly with a hypotonic solution. The cells were then fixed with Carnoy fixative. GTG-banding was carried out using standard protocols [10].

Results

Affymetrix Cytoscan HD contains 2.6 million markers covering all RefSeq genes and 750K SNPs of human genome, and has >99% genotype accuracy. It has been widely applied to ascertain genome abnormalities of a variety of human diseases. However, it is rarely applied to FFPE tissues. To evaluate the usage of Cytoscan HD on clinical FFPE samples, eight cases of lymphoma FFPE tissues were micro-dissected and analyzed through Affymetrix Cytoscan HD chips. The frozen counterparts of these cases were similarly analyzed. Karyotype analyses were performed on all these cases for validation purpose. As shown in Table 1, karyotype abnormalities in 24 of 51 loci had complete matches with frozen tissue copy number analyses. Twenty-two loci from Karyotype analyses had significant overlapped regions detected by frozen tissue CNV analysis. Overall, this represents 90.2% (46/51) concordance between Karyotype and Cytoscan HD analyses. Five loci of Karyotype analyses, however, did not concord with CNV analysis from both frozen and FFPE tissues analyses. These differences may result from heterogeneity of the tumor samples that some genome abnormalities appear in only a fraction of tumor cells.

Most of the clinical samples are in the form of formalin-fixed and paraffin-embedded tissue blocks. The dependence of array analysis on fresh frozen tissues significantly limits its application in clinical setting. To investigate whether FFPE tissues are suitable for cytoscan HD analysis, DNA from the matched FFPE tissue samples was extracted. Similar Affymetrix Cytoscan HD analyses were performed. Cel files of FFPE tissues from 100 normal individuals were used as baseline to calculate the copy number of genome fragments of these FFPE samples. CNV was determined by p<0.05 with at least 25 markers and a minimal length of 500 Kb. As shown in Tables 1–2 and figure 1, the concordant
rates based on CNV length between arrays from the matched FFPE and frozen tissues ranged from 82% to over 99%. Seven of these FFPE blocks are over 1 year old and one is 3 years. The average concordant rate for 1 year old samples is 94.1%, while the rate for the 3 year old sample is 92%. This suggests that the quality of the assays is stable, and is probably not adversely affected by the age of the fixed tissues for at least 1–3 years. When CNV calling was limited to regions that cover at least one gene, we found that 482 genome segments were either deleted or amplified in at least one of the 16 samples. Among these segments, 68 of 86 segments that were determined as deletion in FFPE samples matched the same callings from fresh frozen tissue counterparts, while 352 of 365 segments that were determined as amplification matched those from the fresh tissues (Table 3). The results confirmed a strong correlation between FFPE and fresh frozen tissues (87.1%, Pearson correlation coefficient = 0.72, p \leq 10^{-16}). Interestingly, when CNV results of FFPE were matched with Karyotype studies, the results were extremely similar to those between frozen CNV and Karyotype. There is no statistically significant difference in terms of accuracy. In fact, a slight improvement (25/51 complete match versus 24/51 frozen) was seen due to detection of X chromosome gain in a case that was missed in frozen tissue.

To validate the CNV analysis at individual gene level, FISH assays were performed on all 8 cases of lymphoma using probes specific for BRAF and CITED2. Four samples of lymphoma were found to have gain of BRAF gene by CNV analyses in both frozen and FFPE tissues. Each of these 4 cases was found to have similar amplification of BRAF in the FISH assays, while the other 4 CNV neutral samples were found to have copy number near diploid condition in FISH. The concordant rate between the CNV calls from FFPE or frozen samples and the FISH results from the

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**Figure 2. FISH validation of copy number variation detected by Cytoscan HD analysis.** (A) Representative images of FISH analysis using probes specific for BRAF (7q34, red) and centromere of chromosome 7 (green). Left, normal diploid control, right-case 4 lymphoma (BRAF amplified). (B) Representative images of FISH analysis using probes specific for CITED2 (6q23.3, red) and centromere of chromosome 6 (green). Left, normal diploid control, right-case 2 lymphoma (CITED2 deleted).

doi:10.1371/journal.pone.0092820.g002

**Table 4. Correlation of CNV callings and FISH on BRAF and CITED2.**

| Genes | Cases | CN by frozen tissue | CN by FFPE | CN by FISH |
|-------|-------|---------------------|------------|------------|
| BRAF  | 1     | 3                   | 3          | 2.8*       |
|       | 2     | Unchanged           | Unchanged  | 2.1        |
|       | 3     | Unchanged           | Unchanged  | 1.9        |
|       | 4     | 3                   | 3          | 2.9*       |
|       | 5     | Unchanged           | Unchanged  | 2.2        |
|       | 6     | Unchanged           | Unchanged  | 1.9        |
|       | 7     | 3                   | 2.5        | 2.7*       |
|       | 8     | 3                   | 3          | 3.1*       |
| CITED2| 1     | Unchanged           | Unchanged  | 2.1        |
|       | 2     | 1                   | 1          | 1.3*       |
|       | 3     | Unchanged           | Unchanged  | 1.9        |
|       | 4     | Unchanged           | Unchanged  | 2          |
|       | 5     | Unchanged           | Unchanged  | 2.2        |
|       | 6     | 1.4                 | Unchanged  | 1.6*       |
|       | 7     | Unchanged           | Unchanged  | 1.8        |
|       | 8     | Unchanged           | Unchanged  | 2          |

CN-copy number;
*p < 0.01.
doi:10.1371/journal.pone.0092820.t004
matched samples reaches 100% (8/8, Table 4 and figure 2). CITED2, a transcription modulator essential for glycolytic metabolism for adult hematopoietic stem cells and potential tumor suppressor [11,12], was found deleted in 2 cases of lymphoma by CNV analysis in frozen tissues. On the other hand, CNV analysis in the matched FFPE tissues suggests loss of CITED2 in only one sample. FISH assays using a probe specific for CITED2 found loss of one copy of CITED2 gene in both cases where deletions were also indicated by Cytoscan HD chip analyses of frozen tissues (Table 4 and figure 2). However, 55% (117/212) of the counted cells in one of the cases (case 6) in the FISH assay do not contain deletion of CITED2. The large dilution by the diploid cells in this sample may result in a negative result in FFPE CNV analysis.

Despite the high level statistical CNV concordance between FFPE and frozen tissues, a moderately higher level of fluctuation of copy number for genome deletion for FFPE tissues is 1.44. This magnitude of deletion is significantly less than that from frozen tissues (1.29, p = 6.7 × 10^-13). As a result, CNV analysis from FFPE tissues could be less sensitive. Despite this mild drawback, excellent CNV concordance between FFPE and frozen samples was evident in most of the CNV regions, particularly those CNV loci with large DNA fragment abnormalities (figure 1).

**Discussion**

The current methodologies to detect chromosome abnormalities include Karyotyping using Giemsa staining of chromosomes, high throughput array analysis of single nucleotide polymorphism and DNA copy number, and whole genome sequencing. The first two are the most commonly used methods to determine the copy number of genome fragments, while the last one might be highly precise but expensive. Array genome copy number analysis offers a high resolution alternative to Karyotyping assay. However, its clinical application is limited due to the requirement of high quality DNA. Most clinical specimens, however, are stored in the form of formalin-fixed and paraffin-embedded tissue blocks. The DNA from FFPE is highly fragmented and cross-linked. This produces a significant challenge in using FFPE tissues for high resolution genotyping analysis. Our method using FFPE tissues to analyze chromosomes shows an average of concordance of 93.8% between FFPE and fresh frozen tissues. It suggests that FFPE Cytoscan HD analysis is readily applicable to clinical setting.

**Table 5. Comparison of methods analyzing CNV using FFPE tissue.**

| Loci | Thompson et al | Little et al | Tuefferd et al | Lips et al | Oosting et al | Soroush et al | Yu et al |
|------|----------------|--------------|----------------|-----------|--------------|------------|---------|
| Array Type | Affymetrix | GenomePlex | Affymetrix | Illumina | Illumina/Affymetrix | Affymetrix | Affymetrix |
| Platform | Affymetrix | GenomePlex | Affymetrix | Illumina | Illumina/Affymetrix | Affymetrix | Affymetrix |
| Number of Loci | 10000 | 5623 | 1.85 million | 5861 | 5861/10000 | 40161/1.85 million | 2.6 million |
| CNV FFPE vs. Frozen | NE | NE | 53% | NA | NE | NA | 93.80% |
| CNV FFPE vs. karyotype | NE | NE | NE | NE | NA | NA | 91% |
| FISH validation rate | NE | NE | NE | NA | NE | NA | 93.80% |

**NE-Not examined; NA-Not applicable.**

doi:10.1371/journal.pone.0092820.t005

Studies using FFPE tissues to analyze copy number variation had been previously attempted on Affymetrix 10K and SNP6.0 chips, GenomePlex aCGH, Illumina beadArray and Agilent 244K chips (Table 5) [13–19]. However, many of these studies lack direct validation using other methodologies. In one study, many of these studies lack direct validation using other methodologies. In one study, high noise level on CNV analysis was found in FFPE samples when using Affymetrix 6.0 chip. Only 53% concordant rate was found between the matched frozen and FFPE samples [15]. In another study, only selected concordance analyses were performed on selected regions of FISH and Agilent 244K chips using FFPE tissues [14]. The study concluded complementary roles of between aCGH and FISH analyses. Both analyses were performed on FFPE tissues. Recently, a FFPE OncoScan service was developed by Affymetrix Inc to use Molecular Inversion Probe technology to detect CNV of oncogenic hot spots [20,21]. The signal-to-noise ratios were reduced even using FFPE tissues more than 5 years old. However, these studies lacked direct validation comparison between FFPE and matched frozen tissues. Thus, the fidelity of CNV callings was not determined. Another study using optimization of universal linkage system labeling to analyze 3 cases FFPE and matched frozen tissues. They found a good correlation in 2 cases (Pearson correlations 0.54–0.58), but found poor correlation in the other case [22]. To our knowledge, this is the first report that shows high concordance in CNV analysis between FFPE and frozen tissues using Affymetrix Cytoscan HD chip. The CNV results from FFPE, not only matched well with those from frozen tissues, but were also largely validated by cytogenetic karyotyping and FISH analyses. The Cytoscan HD FFPE analysis holds promise for being widely used in solid tumor and hematological diseases diagnosis. It may be also useful in differential diagnoses of hereditary diseases.

**Acknowledgments**

We thank Song-Yang Zheng and Kathleen Cieply for technical support in the study. Sarah Gibson provided pathology support in identifying lymphoma regions. We also thank Steven Swedlow and George Michalopoulos for frank and constructive comments on the manuscript.

**Author Contributions**

Conceived and designed the experiments: JHL. Performed the experiments: YPY AM YD GT. Analyzed the data: YPY YD GT. Contributed reagents/materials/analysis tools: YD GT. Wrote the paper: JHL.
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