Directional Genomic Hybridization: An Improved Biomarker for Radiation Exposure

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ABSTRACT

Cytogenetic biomarkers have been a preferred choice for retrospective estimation of radiation exposure because they are sensitive, quantifiable, and relevant to biological effects of concern. The most commonly used involve the measurement of dicentrics and symmetrical translocations. Both have shortcomings that become increasingly problematic for assays carried out at long times after radiation exposure. Dicentrics in samples from peripheral blood lymphocytes decrease with time after exposure ($t_{1/2}$ 1-2 years), and in the case of more stable, symmetrical translocations, the background levels are 10-fold higher and increase with age. Another aberration type, inversions, result from exchanges *within* a chromosome that reverse the orientation of the broken segment. We developed an approach based on directional genomic hybridization (dGH^{TM}) that facilitates detection of inversions with a greater than 10 fold improvement in resolution over existing techniques, allowing the detection of 1Mb or smaller inversions. Bioinformatics guided design of sequence- and strand-specific probe sets, which when coupled with single-stranded hybridization, produce *chromatid* – rather than chromosome – paints. Inversions register simply as a signal switch from one sister chromatid to the other in the inverted region. Importantly, like chromosome paints, *chromatid* paints also reveal translocations and dicentrics. Modeling suggested that inversions should be more common than translocations after densely vs. sparsely ionizing radiation exposures. We irradiated human cells with high LET heavy ions (densely ionizing) or low LET gamma rays (sparsely ionizing) and, using chromatid painting, compared the dose-response yields for induction of inversions, translocations and dicentrics. As predicted, the slope of the dose-response curve following heavy ion irradiations was steeper, and the yields per unit dose for inversions were higher than for either translocations or dicentrics. In another illustrative application, chromatid painting of orangutan cells revealed an inversion that presumably occurred during karyotype evolution of mammals. Together, our results demonstrate that inversion detection is useful for a variety of applications and can be further developed for use as a sensitive tool to measure past exposure to ionizing radiation and/or other clastogens. Funding for this work was provided by NASA (NNX09CE42P; NNX10CB05C) and NIAID (R01AI080486-02).

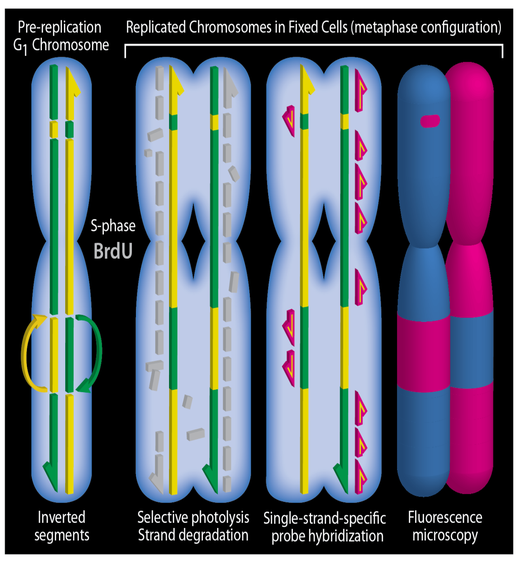

Figure 1. How dGH inversion detection works. Nucleotide analogs-BrdU/C are incorporated during one replication cycle, and each nascent strand becomes photo-labile, allowing it to be selectively degraded (CO-FISH). For the purposes of *in situ* hybridization, this results in a metaphase chromosome whose sister chromatids are single-stranded and complementary. Because inverted DNA sequences reverse their 5'→3' orientation, fluorescently-tagged *single-stranded* probes hybridize to the opposite chromatid. For large inversions, the signal switch is accompanied by a corresponding loss of signal on the opposite chromatid; for small inversions, the loss of signal is not always observable **(Fig. 2 b(small) and c(large)).** The probes are designed to unique DNA *only* and therefore each probe binds to only one location on one chromosome. See **Ray et al., Chromosome Research 21:165, 2013**.

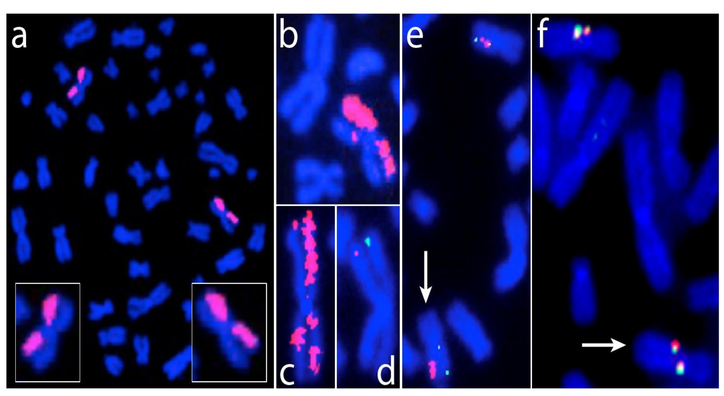

Figure 2. *Chromatid* painting. (a) Directional, fluorescently-tagged (red) *single-stranded* probe sets to unique sequences along one chromatid of human chromosome 3 are both chromosome- and *chromatid*-specific (insets are higher mag.). (b,c) Radiation-induced small and large inversions, respectively. (d) A simulated 6kb inversion (red) on one chromatid of a normal unirradiated cell. The region is flanked by complementary probe sets (green), which hybridize to the opposite chromatid. (e) Targeted high-resolution, two-color detection of known inversions associated with *inv(3)(q21q26)* in AML, and (f) RET/PTC1 *inv(10)*(q11q21) associated with radiation-induced thyroid cancer; arrows depict inverted homologs.

Methods

Cells Two lineages of normal human fibroblasts were used in this study; a mortal strain (NHDF 4012, Clonetics) and the hTERT-immortalized BJ1- cell line, both of which have stable diploid karyotypes. The nucleotide analogs BrdU/BrdC were added (5.0 and 1.0 µM, respectively) and cultures incubated to the first mitosis (22 hours). Colcemid (0.1 µg/ml; KaryoMax, Gibco) was added during the final four hours, after which mitotic cells were harvested and spreads prepared by standard cytogenetic techniques.

For lymphocytes, approximately 10 ml of peripheral blood was drawn from healthy volunteers into heparinized Vacutainers. Three ml of blood was added to each of three 15 ml centrifuge tubes and irradiated. After irradiation, the samples were immediately added to 27 ml blood culture media (KaryoMax, Gibco) in T25 flasks. The flasks were incubated for 13 hour prior to BrdU/BrdC addition. After a total of 53 hours Colcemid was added for two additional hours, mitotic cells were harvested and spreads prepared as above.

Irradiations Fibroblasts and lymphocytes were irradiated with low LET ^{137}Cs gamma-rays in Shepherd Mark 1 irradiators. All exposures were acute and desired doses were attained in less than 5 minutes.

High LET irradiations (^{56}Fe 600 MeV/n – LET 174 keV/µm; ^{16}O 78 MeV/n – LET 57 keV/µm) were performed at the NASA Space Radiation Laboratory (NSRL), Brookhaven National Laboratory, and done in the plateau region of the Bragg curve (i.e., in track-segment mode). Tubes containing cell suspensions were secured in foam holders and placed perpendicular to the beam. All exposures were acute and desired doses were attained in less than 5 minutes.

Chromatid painting Slides with metaphase chromosome spreads were pretreated and hybridized as previously described (Ray et al. 2013). Briefly, slides were stained with Hoëscht 32258, nascent BrdU/BrdC-substituted DNA strands were preferentially nicked by exposure to 365 nm UV light, then removed using exonuclease III (ExoIII). A chromosome 3-specific chromatid paint labeled with Cyanine3 was hybridized for 3 minutes at 73˚C, then incubated overnight in a humidified chamber at 37˚C. After hybridization, the slides were washed five times in 2×SSC at 42 ˚ C for 15 min each. Slides were rinsed in PN buffer, counterstained with DAPI/antifade (18µl; VectorLabs) and coverslipped.

Image capture and analysis was performed on an Olympus Bx41 microscope outfitted with fluorochrome-appropriate excitation/barrier filters, running Metavue 7.1 software and equipped with a Photometrics CoolSNAP ES2 camera. Efficiency of single probe set hybridization (i.e., how often strand-specific signals were observed on chromosome 3) was routinely greater than 90%.

Scoring Metaphase chromosome spreads were selected based on efficiency of chromatid painting; i.e., a Cyanine3-labeled chromatid (red) on one side and DAPI-stained (blue) on the opposite chromatid. Metaphase chromosome spreads exhibiting partial or incomplete chromatid painting (i.e., signal was present on both chromatids of chromosome 3), were not considered. Chromosome 3s that were folded or twisted were also not scored. In a few cases (<5%), it was possible to score only one chromosome 3 and not the other in a metaphase spread; selection was done without bias for the presence or absence of an inversion. Assuming a Poisson distribution, the standard error of the mean was calculated by taking the square root of the inversion frequency divided by the square root of the number of chromosomes scored.

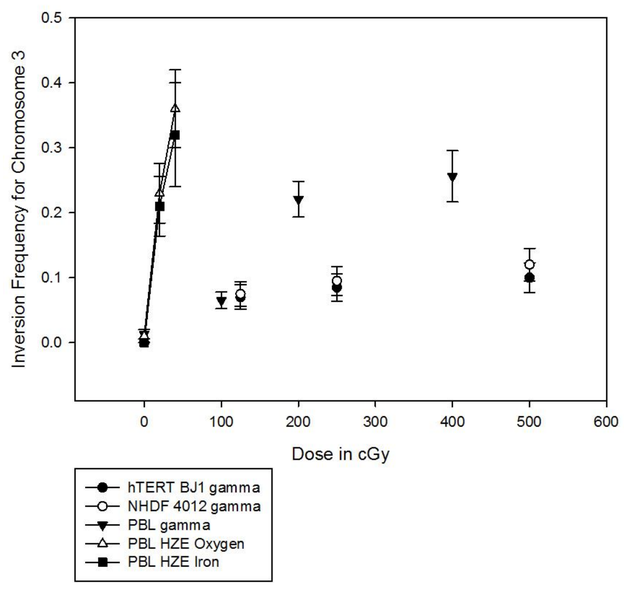

Figure 3. HZE(highLET) versus gamma(lowLET) radiation-induced inversion frequencies. Dose responses were evaluated using a *chromatid*-3-specific paint in normal human fibroblasts (HSF and BJ1) and lymphocytes (PBL) following low LET (^{137}Cs gamma-rays) or high LET (1GeV/n ^{56}Fe; 78 MeV ^{16}O) exposures.

Chromosome 3 contains 6.6% of the DNA in the human genome. To give some perspective, only about one tenth of one per cent of the genome was covered with this chromatid paint (195 fluorescent spots spaced at ~1Mb intervals). Nevertheless, as can be seen in Figure 2, this relatively sparse coverage produced bright composite chromatid paints capable of producing the dose responses shown in Figure 3. It is therefore reasonable to expect that as the coverage of chromatid paints is increased, the resolution of dGH will improve in terms of its ability to discern inversions of smaller and smaller size. This, of course would also increase the yields of inversions per unit dose, making the system a sensitive, easy to score and objective method to score previous exposure to agents that cause dsDNA breaks, such as ionizing radiation.

Another striking example of dGH use to detect radiation exposure involves a highly specific chromosomal inversion that occurs in radiation-induced thyroid cancer **(dGH assay shown in Fig. 2f).** The RET/PTC1 inversion (inv(10)(q11q21) occurs between the RET oncogene and the H4 gene (CCDC6) on chromosome 10 and has a known association with the induction of papillary thyroid cancer (PTC). The rearrangement always occurs in the same two introns of the participating genes, allowing precise positioning of dGH probes near the breakpoints. Caudill et al. (2005) used a human thyroid cell line, HTori-3, to demonstrate a dose-dependent induction of the RET/PTC1 inversion using RT-PCR and primers specific to the mRNA fusion product. They noted a delay of nine days before they could detect a relatively rare signal. We used these same cells (kindly provided by Dr. Nikiforov) to develop and then test a specific RET/PTC1 assay by determining the frequency of the inversion at two time-points after gamma ray exposure, immediately after exposure and after a delay of 70 population doublings.

Immediately Dose(Gy)	#RET/PTC1per cell
0	0
0.25	0
0.5	2(0.02)
1.0	1(0.01)
2.5	1(0.01)

Cells after 70 PD Dose(Gy)	#RET/PTC1 per cell
0	0
0.25	1(0.01)
0.5	6(0.06)
1.0	10(0.10)
2.5	15(0.15)

There was a barely perceptible, immediate dose response and then the frequency of the inversion increased after 70 population doublings. The accumulation of the inversion with cell division suggests that the fusion gene is selected for during *in vitro* culturing.

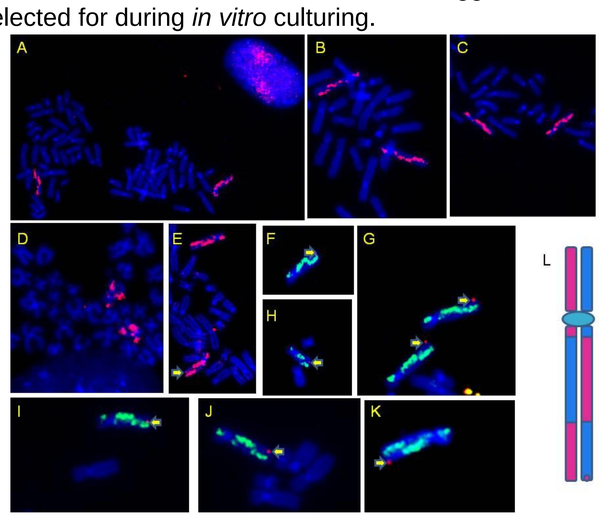

Figure. 4. Synteny application of human chromosome 3 chromatid paint to orangutan fibroblasts. A.-E. The chromatid paint was labeled with Cyanine3**. F.-K.** The paint was labeled with fluorescein and a complementary control spot (arrow) was labeled with Cyanine3. A large recurrent inversion was observed, as expected. **L.** Preliminary consensus result.

dGH Probe Applications

- **Contig orientation and location**
- **Discovery of new inversions**
  - **Measures individual cells**
  - **Recurrent inversions in cancer**
  - **Recurrent inversions in idiopathic diseases**
- **Diagnostic assays**
  - **Pinpoint, objective, and easy to use**
- **Biodosimetry assay**
  - **Sensitive indicator of genetic damage**
- **Biological mechanisms**
  - **Inversions over time**
- **Synteny**
  - **Inversions and evolution**

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