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Directional genomic hybridization for chromosomal inversion discovery and detection

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Abstract Chromosomal rearrangements are a source of structural variation within the genome that figure prominently in human disease, where the importance of translocations and deletions is well recognized. In principle, inversions—reversals in the orientation of DNA sequences within a chromosome—should have similar detrimental potential. However, the study of inversions has been hampered by traditional approaches used for their detection, which are not particularly robust. Even with significant advances in whole genome approaches, changes in the absolute orientation of DNA remain difficult to detect routinely. Consequently, our understanding of inversions is still surprisingly limited, as is our appreciation for their frequency and involvement in human disease. Here, we introduce the directional genomic hybridization methodology of chromatid painting—a whole new way of looking at structural features of the genome—that can be employed with high resolution on a cell-by-cell basis, and demonstrate its basic capabilities for genome-wide discovery and targeted detection of inversions. Bioinformatics enabled development of sequence- and strand-specific directional probe sets, which when coupled with single-stranded hybridization, greatly improved the resolution and ease of inversion detection. We highlight examples of the far-ranging applicability of this cytogenomics-based approach, which include confirmation of the alignment of the human genome database and evidence that individuals themselves share similar sequence directionality, as well as use in comparative and evolutionary studies for any species whose genome has been sequenced. In addition to applications related to basic mechanistic studies, the information obtainable with strand-specific hybridization strategies may ultimately enable novel gene discovery, thereby benefitting the diagnosis and treatment of a variety of human disease states and disorders including cancer, autism, and idiopathic infertility.

Keywords chromatid painting · chromosomal inversions · Strand-specific hybridization

Abbreviations

a-CGH	Microarray-based comparative genomic hybridization

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BrdU	5′-Bromo-2′-deoxyuridine
CNVs	Copy number variants
CO-FISH	Chromosome orientation fluorescence in situ hybridization
FISH	Fluorescence in situ hybridization

Introduction

Abnormalities associated with chromosome rearrangements have been studied for many years, and an appreciation for their importance emerged in parallel with development of methods that enabled their detection. More than 800 tumor-specific chromosome aberrations, primarily translocations and large deletions, have been identified (Mitelman 2000). Detailed analysis of the breakpoints involved in these structural changes has been instrumental in the discovery of many cancer-related genes and, more broadly, has shed light on underlying mechanisms of carcinogenesis. Particularly illustrative early examples include identification of the 9:22 translocation associated with chronic myeloid leukemia (Nowell and Hungerford 1960; Rowley 1973), and the 8:14 translocation that results in Burkitt's lymphoma (Haluska et al. 1986). These and other notable success stories would not have been possible without corresponding advances in cytogenetic methodologies (Rowley 1990).

Contemporary approaches such as fluorescence in situ hybridization (FISH) and microarray-based a-CGH have expanded our view and appreciation of alterations associated with chromosome aberrations. For example, many apparently balanced translocations are actually complex rearrangements that concurrently involve deletions and duplications (Chen et al. 2010; Manning and Hudgins 2010), as well as inversions (Gribble et al. 2005). Chromosomal inversions are intra-chromosomal rearrangements that result from two breaks occurring within the same chromosome, followed by re-insertion of the broken segment in the opposite (inverted) orientation, and are among the most difficult of structural rearrangements to detect.

Traditionally inversions, and other types of structural rearrangements, have been detected as disruptions to the visual patterns produced by various types of banding—megabase-sized striations distributed laterally along the lengths of chromosomes (Bickmore and Craig 1977)—an approach that severely limits the size of inversion that can be detected. Moreover, because such pattern disruptions do not necessarily visibly alter banding patterns (Savage 1977a, b), and because they are undetectable to whole chromosome painting by FISH, even large inversions can be difficult to detect with regularity (Savage 1977a, b). While it is undeniable that a significant subset of inversions is visible by traditional cytogenetic approaches, it is also the case that many (perhaps even most) remain undetected.

Many of the problems relating to pattern recognition and resolution that plague banding analysis can, in principle, be overcome through more molecular approaches. A case in point is the recent discovery of a (p13.3q24.3) pericentric inversion encompassing practically all of chromosome16 that was identified by transcriptome sequencing, but which otherwise was "cryptic" to alternative methods of analysis (Gruber et al. 2012). The discovery, which involved the concerted effort of several institutions and multiple authors, was important for the additional reason that the inversion encoded a fusion protein (CBFA2T3-GLIS2) that defined a particularly aggressive subtype of pediatric acute leukemia.

It is fair to say that molecular methods face their fair share of challenges regarding inversion detection as well, and that despite significant progress made toward characterizing structural variation using such approaches, our understanding of inversions is still surprisingly limited (Baker 2012). Whereas CNVs can be mapped with great precision, changes in orientation (i.e., phase) of DNA sequences are much more difficult to detect. Techniques like paired-end sequencing have great potential to identify and map inversions across the human genome (Tuzun et al. 2005), but can be limited by possible mis-assembly of reference genomes and the presence of flanking high-density inverted duplications (Feuk 2010).

These issues aside, alternative independent methodologies would be of value for validating and characterizing recurrent structural variants identified by sequencing approaches—as, for example, those recently reported for colorectal cancer (Cancer Genome Atlas 2012) and adenocarcinoma of the lung (Imielinske 2012). Highlighting the potential significance of inversions in the etiology of disease, and at the same time underscoring difficulties in detecting and characterizing them (Antonacci et al. 2009), a majority of variants identified in this latter study (Imielinske 2012) were relegated to the generic classification of "intra-

chromosomal rearrangements." One might well suspect that many of the variants in this class were bona fide inversions.

Lastly, it should be noted that sequencing approaches are not particularly well suited to addressing the cellular heterogeneity typical of solid tumors since this would ostensibly involve characterization of abnormalities among mosaic sub-populations. This would also apply to single cell sequencing methodologies (Falconer et al. 2012), which are impractical for use on more than a few cells. For similar reasons, strictly genomics-based approaches would be of dubious value in constructing quantitatively meaningful cellular dose responses—as used for the purpose of risk assessment following exposure to genotoxic agents—because this requires the collective assembly of data from of many cells, each individually assessed for damage.

There exists, therefore, a need for methodologies of chromosomal inversion detection that provide the higher resolution of molecular approaches, while still retaining one of cytogenetics' most powerful assets—its ability to provide an unbiased view of the entire genome on an individual cell basis. Here, we introduce the directional genomic hybridization methodology of *chromatid painting*—not to be confused with the standard practice of *chromosome* painting—that promises to circumvent many of the aforementioned limitations associated with inversion detection. Chromatid painting evolved from the strand-specific hybridization concept of chromosome orientation FISH (CO-FISH; Bailey et al. 2004a, b, c; Goodwin and Meyne 1993; see Fig. 1). Due to the requirement for single-stranded probes however, the methodology was limited to the interrogation of repetitive centromeric or telomeric sequences with simple oligonucleotide probes, where it was used to study a variety of cytogenetic phenomena including: lateral asymmetry (Goodwin et al. 1996); leading- versus lagging-strand telomeres (Bailey et al. 2001); telomere-DNA double strand break fusions (Bailey et al. 2004a, b, c); recombination within sub-telomeric and telomeric regions (T-SCE; Bailey et al. 2004a, b, c;

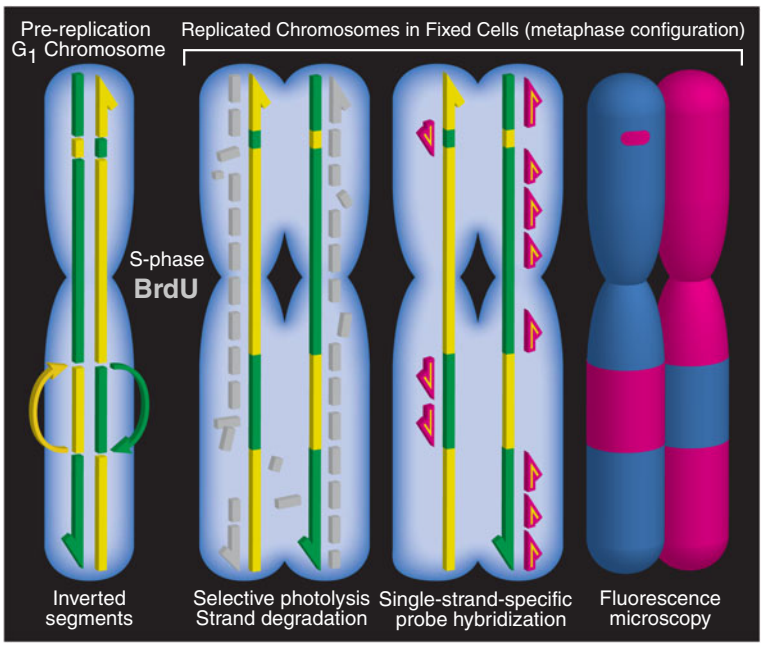

Fig. 1 *Chromatid painting and inversion detection*. Following S-phase, each complementary polynucleotide strand from a G_1 chromosome segregates into respective sister chromatids at metaphase. When BrdU is incorporated during DNA synthesis, each nascent strand becomes photo-labile, allowing it to be selectively degraded. 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 must reverse their 5′→3′ orientation in order to preserve polarity, individual strands within the inversion are obligated to "switch places" between the sister chromatids; therefore, hybridization of fluorescently tagged directional single-stranded probes to unique sequences along either chromatid results in a microscopically visible signal that "switches" from one chromatid to its sister. For large inversions, the signal switch is accompanied by a corresponding lack of signal on the opposite chromatid; for small inversions, the unlabeled segment is often obscured by the brightness of the fluorescent signal

Cornforth and Eberle 2001); and mammalian telomere replication timing (Zou et al. 2004).

With availability and refinement of the human genome database came the ability to utilize bioinformatics-based design schemes for construction of single-stranded oligonucleotide probes to unique sequences along the length of a given chromosome that were also similar with respect to their absolute 5′ pter→3′qter directionality (orientation), an advancement that greatly expanded the potential of strand-specific hybridization strategies. For example, by pooling together of many such unique sequence, strand-specific probes it became possible to effectively "paint" individual whole chromatids of a given chromosome, or any specific region thereof. In so doing, changes in directionality (inversions) occurring anywhere along the length of a painted chromosome readily reveal themselves as an abrupt "switch" in the location of hybridization signal—from one sister chromatid to the other. This cytogenomics-based approach of achieving strand-specificity provides investigators a new tool for inversion discovery and high-resolution inversion detection.

Materials and methods

Cell culture

Human cells (normal fibroblasts and lymphocytes; Kasumi-4 leukemia cell line; HTori-3 immortalized thyroid cells), and chimpanzee, gorilla, and orangutan fibroblasts, were cultured for a single cell cycle in complete media containing 5.0 μM 5-bromo-2-deoxyuridine and 1.0 μM 5-bromo-deoxycytidine (BrdU/BrdC; Acros, Chem Impex International). Cells were blocked in mitosis for 2–4 h using Colcemid (KaryoMax, Gibco) at a final concentration of 0.1 μg/ml. Mitotic cells were harvested and dropped onto slides using standard cytogenetic protocols (Bailey et al. 2010).

Directional strand-specific probes

Creation of the human chromosome 3 specific *chromatid* paint began by downloading contiguous DNA sequences from the publically available NCBI genomic database (GRCh37.p2 primary assembly) and masking them using Genetic Information Research Institute software; i.e., repeat sequences were removed. Single-stranded oligonucleotide probes (oligos) were designed to unique sequences and our specifications using ARRAY Designer (version 4.2), and later with proprietary software (KromaTiD Inc.), tiling to small target regions at specified non-overlapping locations along the length of each chromosome 3 contig at ~1-Mb intervals. Oligo design criterion demanded similar directionality and length (~40-mers), as well as uniform melting temperatures (70.0±5.0 °C). More than 17,000 individual oligos were synthesized (Invitrogen), hydrated, and pooled in subsets of 45 for end-labeling with fluorescent dNTP analogs (Cy-3, Fluorescein; General Electric, Perkin Elmer) using terminal transferase (New England Biolabs). Pools of 90 individual labeled oligos constituted a probe set, which together spanned relatively short unique regions (~5–14.5 kb); probe sets were hybridized individually to confirm strand specificity. The complete chromosome 3-specific chromatid paint consisted of 190 probe sets.

In similar fashion, targeted probe sets to both sides of known inversion breakpoints on chromosome 3 (*q21*; *q26*) and chromosome 10 (*q11.2*;*q21*) were also generated. Targeted probe sets were larger in that they consisted of 180–200 individual labeled oligos and they covered larger unique regions (~14–63 kb), modifications that served to increase brightness and visibility of the individual signals. Proximity of the selected probe sets to the specific inversion breakpoints did not exceed 1 Mb.

Single-stranded hybridization pre-treatment

Briefly, slides (25× 75×1 mm) were incubated in PN buffer (sodium phosphate) for 10 min at room temperature, rinsed in phosphate-buffered saline, then dehydrated through an ethanol series (75, 85, and 100 %) for 2 min each. Slides were air dried, stained with Hoechst 33258 (0.5 μg/ml in 2× sodium citrate; SSC) for 15 min in the dark, then rinsed with deionized distilled water (ddH_2O). Slides were air dried, flooded with 2× SSC, coverslipped (1 mm), and exposed to 365 nm ultraviolet light (UV Stratalinker 2400; Strategene) for 35 min, then rinsed in ddH_2O to remove coverslip, air dried, and dehydrated in the ethanol series, as above (Bailey et al. 2010). For use of single-stranded directional probe sets with standard (double-stranded) FISH, slide pretreatment was not performed prior to hybridization.

Strand-specific hybridization

For each pretreated slide, a mixture of hybridization buffer (25.3 μl; KromaTiD), *chromatid* paint (2.25 μl;

KromaTiD), and ddH_2O (2.45 μl) was prepared and heated to 75 °C for 5 min, then pipetted onto pre-treated slides, which were coverslipped and sealed with rubber cement. Slides were heated at 73 °C for 3 min, then transferred to individual hybridization chambers (Corning) and incubated at 37 °C overnight. 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 (18 μl; Vector Labs), 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 ES^2 camera. Efficiency of probe hybridization was routinely greater than 90 %. Number of metaphases scored was dependent on end-point (e.g., recurrent inversions only required 3–5) and treatment (e.g., dose).

Results

Directional genomic hybridization methodology

A prerequisite for achieving strand-specific hybridization is that cells incorporate bromo-deoxyuridine/deoxycytidine (BrdU/BrdC) during a single round of replication, so that sister chromatids are unifilarly substituted (Fig. 1). Prior to hybridization with single-stranded probes, slides are stained with Hoechst 33258, exposed to UV light to nick the DNA at sites of BrdU incorporation, and treated with Exonuclease III to selectively degrade the newly replicated strands (Bailey et al. 2010). For the purposes of subsequent hybridization, this strategy effectively renders the entirety of each sister chromatid a single-stranded target.

Bioinformatics-based approaches were developed and employed to guide the design of sequence-specific, single-stranded oligonucleotides, which were also of similar directionality. We initially reasoned that the orientation of targets along a single contig had the highest probability of being of similar 5′→3′ direction; therefore, the largest contig on chromosome 3q (NT_005612) was selected (Fig. 2a). Probe sets to unique sequences were designed, synthesized, labeled (green), and hybridized to pre-treated human metaphase chromosomes, which produced a strand-specific signal at the predicted location. Consistent contig alignment along the entire length of chromosome 3 was confirmed by designing probe sets to the remaining three contigs, each of which contained 90 individual oligonucleotides that were labeled (red) and hybridized similarly. All produced strand-specific signals at the predicted locations along chromosome 3, and all were on the same chromatid, thereby validating similar 5′→3′ direction of the assembled contigs (Fig. 2b). Extending this cytogenomic strategy, some 17,000 individual (single-stranded) oligonucleotides were synthesized, pooled, and fluorescently labeled to create a complete chromosome 3 *chromatid*-specific paint. Figure 3a illustrates the robustness and specificity of the *chromatid* paint hybridized to a human whole-metaphase spread (effectively single-stranded due to pre-treatment), where hybridization is confined to one, and only one, chromatid of the target chromosome.

Genome-wide inversion discovery

Due to the necessity of maintaining DNA 5′-to-3′ polarity, inverted segments reinsert themselves into chromosomal DNA in the reversed (or opposite) orientation. Because the sequence- and strand-specific probes all possess the same 5′ →3′ directionality (similar orientation) and thus are capable of hybridizing only to complementary stretches of single-stranded chromatids, inversions are revealed as obligatory color switches of signal from one sister chromatid to the other (Fig. 1). Ionizing radiation (IR) is well known for its ability to induce chromosome rearrangements (Cornforth 1998), including inversions (Muhlmann-Diaz and Bedford 1995), and was used here to demonstrate the utility of *chromatid* painting for discovery of novel inversions. Figure 3b, c shows small and large inversions, respectively, following exposure of human cells to IR (gamma rays).

Assuming all probe sets yield visible signals, *chromatid* painting with probes spaced at 1 Mb intervals will reveal any inversion of 1 Mb length or larger. Inversions less than 1 Mb may also be detected if by chance a probe set falls within the inverted region. This happens with a frequency that depends on inversion size. For example, 50 % of 0.5 Mb inversions occurring at random locations produce visible signals; similarly 10 % of 0.1 Mb inversions are detectable. Detection of much smaller inversions is possible with more densely spaced probes. To illustrate this important point, we designed a mock "mini-

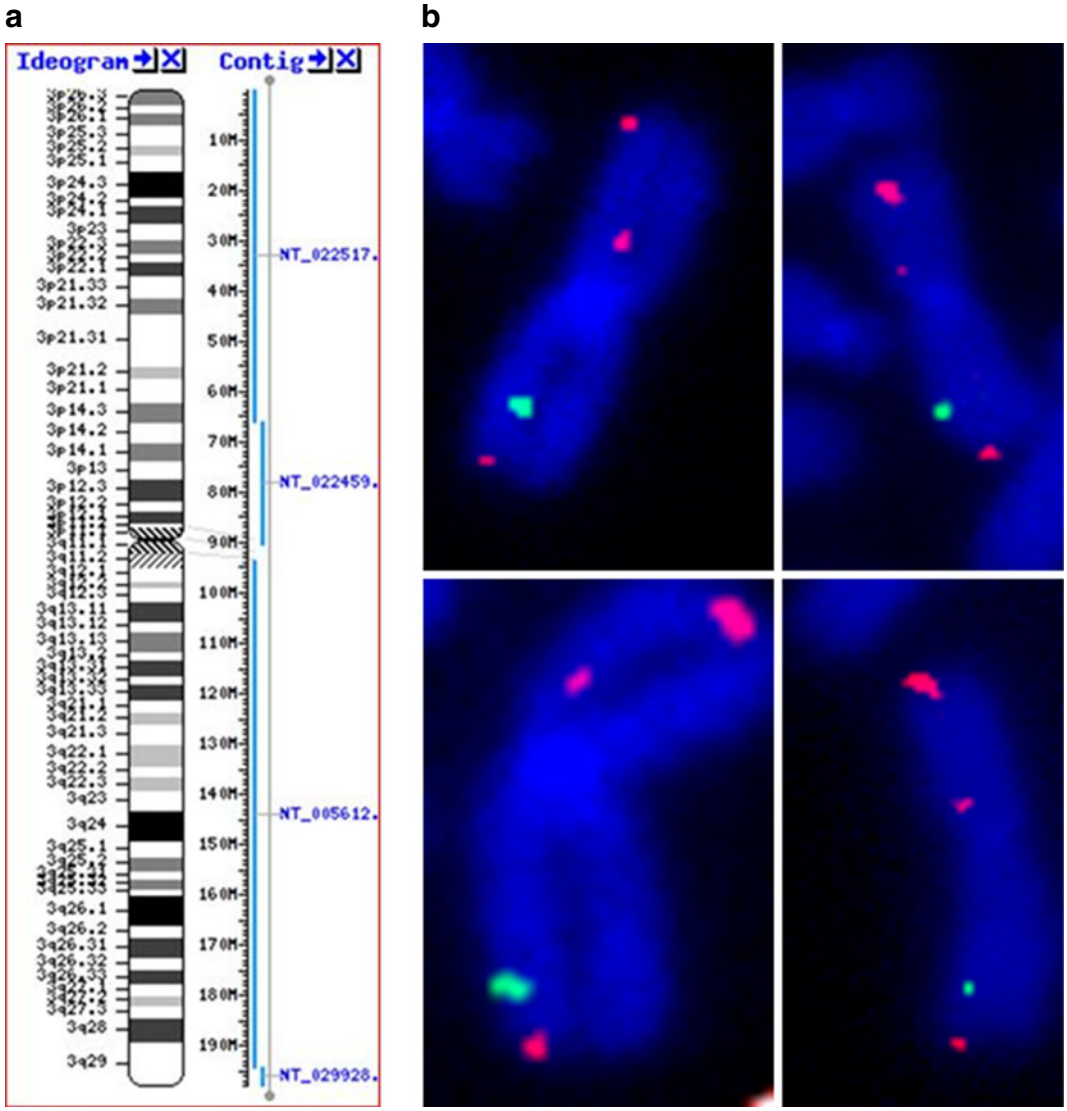

Fig. 2 Confirmation of chromosome 3 contig alignment. **a** Human genome database contig map: http://www.ncbi.nlm.nih.gov/projects/mapview/maps.cgi?taxid=9606&chr=3 **b** Probe sets to all four contigs along chromosome 3 were designed, synthesized, labeled, and hybridized to normal human fibroblasts following strand-specific pre-treatment; one probe set to the large NT_005612 contig (*green*) and probe sets to the other three contigs (*red*) are shown

inversion" within a 10 Mb region of chromosome 3q. Probe sets covering the region were tagged with fluorescein (green); excluded was a 6 kb segment nested within the region. For this segment, oligos were intentionally designed in the reverse orientation, synthesized and tagged with Cy3 (red). This "simulated" 6 kb inversion serves as further validation, provides a useful reference marker, and demonstrates that even quite small inversions are cytogenetically detectable utilizing directional genomic hybridization strategies (Fig. 3d).

High-resolution targeted inversion detection

The ability to detect and characterize inversions can be further augmented using high-resolution, strand-specific probe sets targeted to previously verified or suspected breakpoints. The Kasumi 4 cell line, derived from a patient with chronic myelogenous leukemia (CML), possesses a variant chromosome 3 homolog with a large *q21*; *q26* inversion (Asou et al. 1996). Strand-specific hybridization of Kasumi 4 metaphases with closely spaced probe sets—targeted across the known region of the

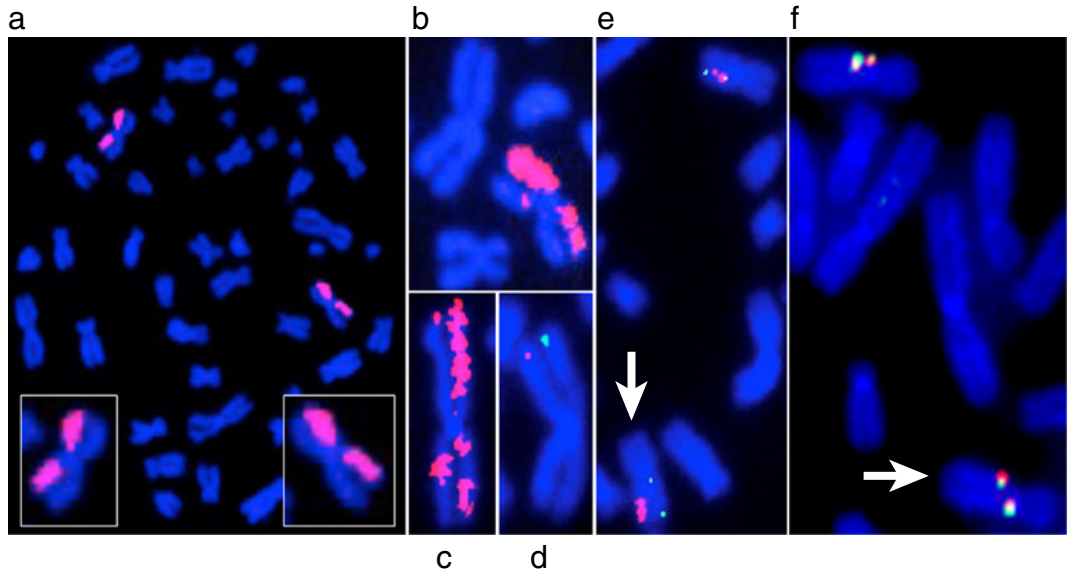

Fig. 3 Inversion discovery and detection with directional genomic hybridization. **a** Directional, fluorescently tagged (*red*) single-stranded probe sets to unique sequences along one chromatid of human chromosome 3 are both chromosome- and chromatid-specific (*insets* are higher magnification). **b**, **c** Ionizing radiation-induced small and large inversions, respectively. **d** A simulated 6-kb 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 inversion breakpoints associated with *inv*(*3*) in CML, and **f** *inv*(*10*) associated with thyroid cancer; *arrows* depict inverted homologues

breakpoints and labeled with different fluorochromes—readily identified the inverted homolog as the one in which the red segment switched to the opposite chromatid (Fig. 3e, arrow). The green signals on its sister chromatid represent probes directed to sequences proximal and distal to the inversion breakpoints. The RET/PTC1 rearrangement associated with radiation-induced papillary thyroid carcinoma provides another noteworthy example as it involves a *q11.2*;*q21* inversion in one homolog of chromosome 10 (Caudill et al. 2005). Here, HTori-3 immortalized human thyroid cell cultures were irradiated and interrogated with strand-specific probe sets targeted to the inverted region on chromosome 10. Consistent with Caudill et al., analysis at time zero revealed a small subset of cells in the exposed population that contained the *inv*(*10*)(*q11.2*; *q21*) rearrangement in one of the homologues, as evidenced by the obvious splitting of the strand-specific signals across sister chromatids (Fig. 3f; arrow).

Other applications of directional genomic hybridization

The human *chromatid*-3 paint also hybridized to closely related hominoid species, specifically chimpanzee, gorilla, and orangutan (Supplementary Fig. 1), supportive of its application in comparative and evolutionary studies. Visible gaps in hybridization signal likely represent repetitive regions, for example around the centromeres, or stretches of sequence not identical to human, as these would not be present in the *chromatid* paint, and therefore are not detected. Consistent with other observations, variation in the state of chromosome compaction also appeared to influence signal intensities. Directional strand-specific hybridization revealed no recurrent inversions in either chimpanzee (Supplementary Fig. 1a) or gorilla (not shown). In contrast, a large inversion was noted in both homologs of orangutan chromosome 3 (Supplementary Fig. 1b).

Chromatid painting facilitates simultaneous detection of *both* intra-chromosomal changes (inversions) and inter-chromosomal changes (dicentrics, translocations). The damage-induced breakage and mis-repair of two chromosomes that results in dicentric formation also produces a compound fragment consisting of the two broken acentric pieces, which also mis-rejoin. These events are clearly visible with *chromatid* painting (Supplementary Fig. 2), where one broken chromosome 3 is involved with an unidentified broken chromosome to form a dicentric chromosome; the associated compound acentric

fragment is definitively identified as it consists of the remaining pieces of chromosome 3 and the other chromosome. *Chromatid* painting concurrently revealed a large inversion (color switch to sister chromatid) in the uninvolved chromosome 3 (Supplementary Fig. 2b).

The advantages of oligonucleotide probes for standard FISH applications have been recently demonstrated (Boyle et al. 2011; Yamada et al. 2011). Noteworthy of such strategies is the superb specificity of the probes themselves, which do not require unlabeled repetitive DNA (Cot1) to block non-specific binding during hybridization. This valuable feature is true of our strand-specific directional probe sets as well, regardless of whether they are being used for (single-stranded) *chromatid* painting, or with standard (double-stranded) FISH for conventional chromosome painting (Supplementary Fig. 3).

Discussion

Utilizing strand-specific hybridization strategies coupled with bioinformatics for sequence- and strand-specific directional probe design, we have developed *chromatid* (as opposed to chromo*some*) painting. This cytogenomics-based methodology offers high-resolution detection of chromosomal inversions on a cell-by-cell basis. Although the current resolution of inversion detection by *chromatid* painting is on average ~1 MB based on spacing of the probe sets, and represents an order of magnitude improvement over traditional banding, this is by no means the limit of resolution that can be achieved. We demonstrate that detection of much smaller inversions is entirely possible with more densely targeted probe coverage, placing chromatid painting among approaches normally considered the purview of high-resolution cytogenetic methods such as interphase FISH (Mancini et al. 2000) or Fiber FISH (Korbel et al. 2007). However, we also note that resolution in terms of inversion detection should perhaps more precisely be viewed as "probability of detection," a more accurate way of viewing the fact that based on spacing of probe sets, smaller and smaller inversions can indeed be detected, but with smaller and smaller probability depending on the location of the breakpoints. Importantly, *chromatid* painting revealed that the spontaneous frequencies of inversions in both normal human fibroblasts and lymphocytes were quite low (<0.3 %), a favorable and critical factor when evaluating dose responses for inversions induced by various genotoxic agents including IR, where medical, environmental, and occupational exposures represent significant health concerns.

The realization of strand-specific hybridization has, at least to date, provided corroborating support for the assembly of the human genome database in terms of it being aligned and oriented correctly, as well as initial evidence that individuals themselves share similar sequence directionality. Analogous experiments conducted during the design of human chromosome 10 strand-specific probes revealed similar orientation of contigs along this chromosome as well (not shown). Interestingly, we also noted that dependent on probe set spacing, fluorescent signals did not always present as uniform visibly contiguous stretches. A possible explanation for this may be related to the variable state of chromatin condensation that chromosomes present throughout metaphase, prompting our speculation that strand-specific probe sets designed at specific and varying intervals may be useful for investigating chromosome compaction.

We provide evidence of the utility of *chromatid* painting strategies for comparative and evolutionary studies, demonstrating their likely potential to complement studies comparing human and distantly related species, as for example was recently reported with the Y chromosome (Hughes et al. 2012). In fact, directional genomic hybridization methodology could be used to develop probe sets to any species with suitable chromosomes whose genome has been sequenced, e.g., equine, canine, feline, and exotic or endangered species. Additionally, unlike conventional chromosome painting, *chromatid* painting facilitates simultaneous detection of *both* intra-chromosomal (inversions) and inter-chromosomal rearrangements (dicentrics, translocations). And like chromosome painting, it is also amenable to multi-color combinatorial schemes of chromosome identification (Speicher et al. 1996). Lastly, the ability to use sequence- and strand-specific probe sets for standard FISH applications greatly expands their use for diagnostic and prognostic purposes.

Although inversions have been detectable at the resolution of traditional cytogenetics (>10 Mb) for many years, there has been a paucity of methods for high-resolution global, unbiased inversion discovery. Therefore, it is largely unknown how common inversions really are, what their size distribution is, and to what extent they are associated with human disorders.

Improved detection of inversions, especially small ones, such as that provided by directional genomic hybridization strategies, stands to facilitate efforts to characterize the contribution of inversions to structural variation in the human genome, thereby aiding investigation of underlying disease mechanisms. Ultimately, novel gene discovery and high-resolution inversion detection have the potential to benefit the diagnosis and treatment of a variety of disease states and disorders including cancer, autism, developmental delay, and idiopathic infertility.

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