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Visualization of Fine-Scale Genomic Structure by Oligonucleotide-Based High-Resolution FISH

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aCGH · Genomic structure · High-resolution FISH · In silico probe selection · Oligonucleotide libraries

Abstract
The discovery of complex structural variations that exist within individual genomes has prompted a need to visualize chromosomes at a higher resolution than previously possible. To address this concern, we established a robust, high-resolution fluorescence in situ hybridization (FISH) method that utilizes probes derived from high complexity libraries of long oligonucleotides (>150 mers) synthesized in massively parallel reactions. In silico selected oligonucleotides, targeted to only the most informative elements in 18 genomic regions of interest, eliminated the need for suppressive hybridization reagents. Because of the inherent flexibility in our probe design methods, we readily visualized regions as small as 6.7 kb with high specificity on human metaphase chromosomes, resulting in an overall success rate of 94%. Two-color FISH over a 479-kb duplication, initially reported as being identical in 2 individuals, revealed distinct 2-color patterns representing direct and inverted duplicons, demonstrating that visualization by high-resolution FISH provides further insight in the fine-scale complexity of genomic structures. The ability to design FISH probes for any sequenced genome along with the ease, reproducibility, and high level of accuracy of this technique suggests that it will be powerful for routine analysis of previously difficult genomic regions and structures.

Molecular cytogenetics is an increasingly popular term as the study of chromosomes moves toward higher resolution through the integration of the larger-scale study of chromosomes with recent fine-scale genomic discoveries [Gray et al., 1992; Speicher and Carter, 2005; Bar-Shira et al., 2006]. Many of the methods for molecular cytogenetics are powered by the recent explosion of genomic information and bioinformatic infrastructure that enables the use of such vast data sets. Array comparative genomic hybridization (aCGH) is now a broadly

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utilized methodology for detecting changes in genomic copy numbers [Albertson and Pinkel, 2003; Schaeffer et al., 2004; Cheung et al., 2005; Lee et al., 2007]. The use of aCGH is increasing our understanding of chromosome abnormalities and revealing structural complexity at a previously unappreciated resolution.

Despite the ability to detect very small DNA abnormalities, many of the fine-scale methods that are pioneering the field of molecular cytogenetics, such as aCGH and next-generation sequencing, lack the full extent of information that is required for cytogenetic analysis. Because average values across a population of cells investigated are represented by these methods, cell-to-cell variation and the presence of mosaicism are difficult to ascertain. Although the use of aCGH and DNA sequencing is expanding our understanding of chromosome abnormalities and copy number variations at a high resolution, complementary methods of a similar resolution that provide the orientation and position of chromosomal structures are necessary to obtain the full details of chromosomal complexities.

One of the most commonly used methods to confirm and further define array findings is fluorescence in situ hybridization (FISH). FISH is the preferred method for validating array findings because chromosomes are visualized within their cellular context [reviewed in Raap, 1998]. In FISH, specific fluorescent labeling of chromosomal DNA is achieved on intact nuclei, which allows for the accurate and precise analysis of both cellular heterogeneity in the population being studied, as well as the chromosomal organization within each cell.

Originally developed as a clone-based method, FISH probes have been generated from genomic DNA, bacterial artificial chromosomes, fosmids, and other sources of template DNA, frequently using random-primed PCR amplification [Raap, 2001; Wang, 2002]. Because most FISH probes currently being used routinely range between 150–300 kb in size, many of the abnormalities detected by aCGH are difficult to visualize using FISH due to their sizes or composition of their genomic regions. The use of specific primers to generate probes from genomic DNA has been suggested to alleviate some of these problems associated with clone-based probe generation [Navin et al., 2006]; however, this process still requires multiple, specific amplification reactions and downstream processing with upfront hands on-time. Other FISH methods that utilize oligonucleotides as probes have been limited to probes targeting repetitive elements [Amann et al., 1990; O'Keefe et al., 1996].

Recently, massively parallel de novo synthesis of oligonucleotide libraries has demonstrated its utility and advantages for many molecular methods [Porreca et al., 2007; Gnirke et al., 2009]. These libraries have been created with up to 55,000 independent oligonucleotide sequences in a single synthesis run, thereby drastically reducing the labor and cost associated with the manufacturing of complexity at these scales. To maximize the freedom to design probes with the greatest flexibility and to minimize the labor required to generate probes, we employed the use of high complexity oligonucleotide libraries as the starting point for probe generation. Genomic data from reference genomes were incorporated to define boundaries for informative and non-informative sequences. Different algorithmic methods for the selection of probe sequences based on in silico predictions were also compared. To test the robustness of this method, we characterized abnormalities detected by aCGH which conventional FISH had difficulty visualizing and validated the methods with a rigorous objective criterion.

Materials and Methods

Selection of Genomic Loci

The initial aCGH microarray assays were carried out by the Molecular Cytogenetic Laboratory (MCL) at ARUP Laboratories using the Agilent aCGH platform employing a 44,000 oligonucleotide custom design; this array design is currently used for detecting copy number changes in the clinical setting. Eighteen specific duplication and deletion sites, ranging in size from 6.7–479 kb were identified and selected as candidate regions for oligonucleotide-based FISH probes (online suppl. table 1, www.karger.com/doi/10.1159/000322717). All coordinates listed are from human genome build 18 (hg18).

Probe Design, Synthesis, and Labeling

Probe sequences were selected in silico from non-repetitive genomic sequences. For simple tiling, probe sequences were chosen by end-to-end tiling oligonucleotides from the 5′ end of the target sequence while maintaining an overlap of 50 bp. For the modified, selective tiling probe design approach, oligonucleotides were tiled at a 10-bp spacing over the region of interest. Melting temperature (T_m) matching was then performed to select a subset of oligonucleotides at a median density of 100 bp overlap. Finally, for both approaches, probes with homology to non-targeted sequences were filtered out.

All in silico selected sequences were sent for massively parallel de novo synthesis, and chemically synthesized oligonucleotide libraries were generated by Agilent Technologies. These libraries were amplified by PCR, followed by the introduction of fluorescent labeling. Labeling was conducted using Kreatech's Platinum*Bright*: Nucleic Acid Labeling Kit. USP spectrum orange with an emission of 565 and spectrum green with an emission of 517 were used as fluorescent dyes.

Control Specimens

Five samples from chromosomally characterized de-identified males analyzed by ARUP Laboratories and not expected to have an alteration at the loci of interest were pooled, dropped on a slide and aged for 30 min at 56°C or overnight at room temperature. The slides were submerged for 2 min in 37°C 2× SSC, after which they were run through an ethanol series of 70, 85, and 100% for 1 min each.

Hybridizations

Each probe was prepared by mixing 40 ng of labeled DNA, 7 μl of Vysis CEP buffer, 0.5 μl of corresponding pericentromeric CEP probe (Abbott Molecular), if available, and water to a total volume of 10 μl. After probe application, a coverslip was sealed with rubber cement. The slide and probe were co-denatured at 78°C for 5 min and hybridized at 37°C for 12–18 h using an Abbott Molecular Thermobrite.

The slides were washed using a SciGene Little Dipper. Three baths were set up with the following solutions: 0.4× SSC/0.3% Igepal at room temperature, 0.4× SSC/0.3% Igepal at 72°C ± 1, and 2× SSC/0.1% Igepal at room temperature. The slides were submerged in the 1st bath for 2 min with slight agitation for 4 s, the 2nd bath for 2 min without agitation, and the 3rd bath for 1 min. Slides were spun dry for 5 min at 1600 rpm and counterstained with DAPI (4′,6-diamidino-2-phenylindole). Chromosome and band localization was done with inverted DAPI visualization (a pseudo-G band pattern), and, in some cases, the use of a pericentromeric CEP probe obtained from Abbott Molecular.

Probe Validation/Assessment

Six slides were evaluated per probe using previously published rigorous criteria [Wolff et al., 2007]. The 1st slide was evaluated for signal intensity, specificity, and sensitivity by scoring 100 metaphase cells. Signal intensity was evaluated on a scale of 1–5 based on the strength of the signal; a value of 5 was given if the intensity was comparable to commercially produced probes for larger regions. If no signal was detected, '1' was entered for signal intensity and no value was entered for the other categories. Specificity was calculated by dividing the number of signals seen at the correct location over the total number of signals seen and the percentages were fitted to a scale of 1–5. Sensitivity was determined by dividing the number of signals seen at the correct chromosomal location over the number of expected signals and the results were fitted to the same scale. The remaining 5 slides served as within and between run reproducibility tests. Each slide was evaluated for signal intensity, specificity, and sensitivity. The reproducibility value was determined by calculating the sum value given to each slide and dividing by the number of slides used (5). The total value given to each probe is the sum of the 4 categories with a maximum score of 20.

Duplication Analysis

To further characterize the 479-kb duplicated region of 15q13.3 (29,818,104–30,297,359), two additional probes were designed, covering the 5′ and 3′ ends of this region with probes of 23 kb and 56 kb, respectively. The probes were labeled with either orange or green and combined for multi-color FISH analysis on 2 duplication positive samples.

Results

Performance of oligonucleotide-based FISH was tested on 18 genomic loci using cells from a pool of 5 normal individuals. The sizes of the loci range from 6.7 to 479 kb, but after removing the repetitive sequences and sequences with high homology to non-targeted regions, probe coverage ranged between 3.6 and 144 kb (online suppl. table 1).

Probes with Simple Tiling Designs

We determined the signal intensity, specificity, sensitivity, and reproducibility values for probes designed by simple tiling using a scoring system ranging from 1–20 as described in Materials and Methods. The simple tiling-based probe designs produced scores >15 for 16 of the 18 loci (89%; online suppl. table 2). All but one of these regions have a combined probe coverage of <60 kb after repeats and sequences with high homology to non-target regions were removed. One region of 479 kb (15q13.3; 29,818,104–30,297,359) was included to demonstrate the utility of the probes for larger regions. Although these regions were not specifically chosen because of their location, 10 out of 18 loci were near the telomeric regions (online suppl. table 1).

We demonstrated that high intensity signals could be generated from loci that cover regions as small as single genes with a probe on 2q13, where our probes essentially covered 1 gene (*NPHP1*; fig. 1c). We were able to obtain robust FISH signals by tiling only 33% of the region (online suppl. table 1), demonstrating how specificity and high signal to noise can be achieved by tiling through only the most informative elements of the target region.

One locus (6p25.3; 174,615–284,943) showed non-targeted but very specific hybridization at a secondary locus on chromosome 16. Interestingly, when this probe was tested in 2 independent laboratories on control cells from 2 different individuals, the secondary locus did not show consistent hybridization. Further investigation of the 6p25.3 locus showed that this region has been reported to exhibit high frequency copy number polymorphism with evidence of frequent inter-chromosomal events associated with chromosome 16 [Kidd et al., 2008]. Therefore, we believe the secondary signal on chromosome 16 is likely to be the site of copy number polymorphism. Inter-chromosomal copy number variation has recently been reported for other loci [Conrad et al., 2010]. FISH results on additional control samples showed all of the following: double hybridization signals from chromosome 6 alone, double hybridization signals from chromosome 6 with a

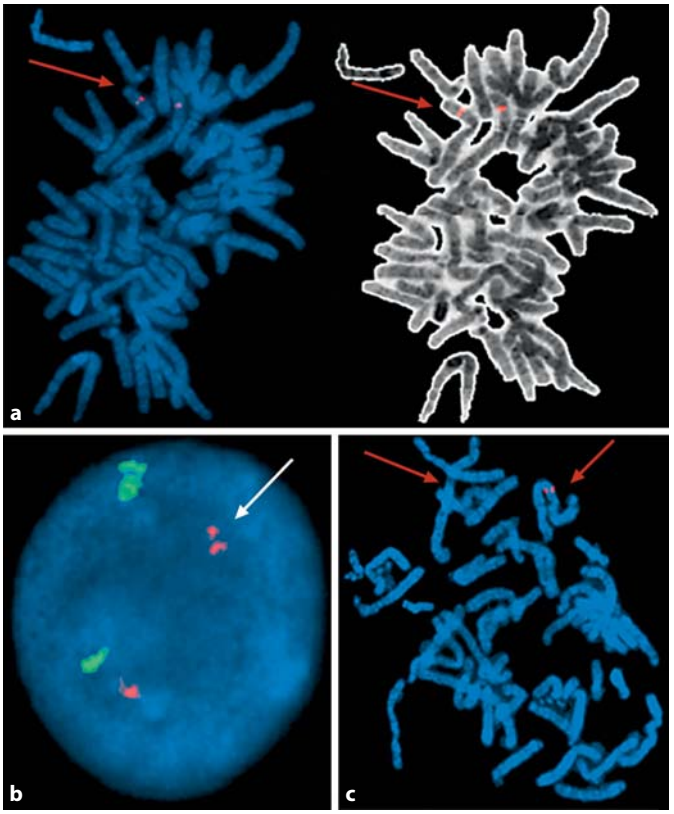

Fig. 1. Fine-scale visualization of genomic structures by oligonucleotide-based FISH. **a** A 6.7-kb region at 6p22.2 (110,219,652–110,316,643) is detected using oligonucleotide-based FISH, shown by the red signals. The same FISH image is shown with DAPI counterstain (left), and inverted DAPI stain or 'pseudo G-banding' confirming the chromosomal location (right). Arrows indicate chromosome 6. **b** Confirmation of a 79.5-kb 9q34 duplication (138,270,360–138,349,890) in one chromosome 9 as indicated by 2 red signals (arrow). Pericentromeric regions of chromosomes 9 are displayed by green signals. **c** Deletion of the *NPHP1* region in 2q13 (110,219,652–110,316,643) is confirmed in metaphase cells by red-labeled FISH probes and DAPI counterstain. Arrows indicate chromosomes 2, with the chromosome to the left showing the deletion (no red FISH signal).

Table 1. Selective tiling probes show improved or similar performance over simple tiling probes

Locus	Region size (kb)	Tile size (kb)	Simple tiling (score)	Selective tiling (score)
6p22.2	6.7	3.6	1	15
15q13.3	479	144	20	20
16p13.3	29	16	19	20
16p13.3	16	10	19	20
17p13.3	45	25	20	20
4p16.3	63	8.6	17	18
Xq27.1	31	6.8	1	14

single hybridization signal from one copy of chromosome 16, and double hybridization signals from both chromosome 6 and chromosome 16, in various, independent control samples (online suppl. fig. 1).

Five loci in this data set were tiled with a probe coverage of <10 kb in size (tile size; online suppl. table 1). Of these 5, high-performing reproducible results could be achieved for 3 loci. To improve the signal quality for the remaining 2 loci, we developed a refinement on our selection criteria for probe sequences.

Probes with Selective Tiling Designs

In addition to the 2 loci that did not perform well with simple tiling rules, we selected 7 loci to investigate the effect of selectively tiling oligonucleotides after algorithmic selection based on T_m matching. In order to determine whether size-based effects exist for our selection process, region sizes ranging from 6.7 kb (3.6 kb tiled) to 479 kb (144 kb tiled) were investigated (table 1 and online suppl. table 2). In all 7 out of 7 loci, results remained equally good or improved upon selectively tiling the oligonucleotides that are used to create the probe mixture (online suppl. table 3). A marked improvement was observed for the smallest locus, 6p22.2 (110,219,652–110,316,643), which could not be visualized by the simple tiled probe design. A representative image for the selective tiling designed 6p22.2 probe is shown in figure 1a. Similarly, a detectable signal could only be achieved for Xq27.1 with the selective tiling designed probe; however, this locus showed significant sample-to-sample variation in signal intensity that was not observed for any other locus in this study. In all cases, there was no reduction in performance as a result of selective tiling, such as loss of specificity or reproducibility in any of the loci tested.

Concordance with aCGH Findings

Cells from specimens with abnormalities determined by aCGH were analyzed by oligonucleotide-based FISH probes designed for specific aCGH-identified aberrations for 10 of the 18 loci. Two cases with deletions and 8 cases with duplications were successfully confirmed, demonstrating 100% concordance between aCGH and FISH findings (online suppl. table 4). All duplications were indicative of a 3-copy state from aCGH signal intensity measurements and appeared to be tandem by FISH; no duplicated copies were identified elsewhere in the genome. Representative images for 2 regions are shown in figure 1b (duplication of 9q34; 138,270,360–138,349,890) and 1c (a deletion of the *NPHP1* region at 2q13; 110,219,652–110,316,643).

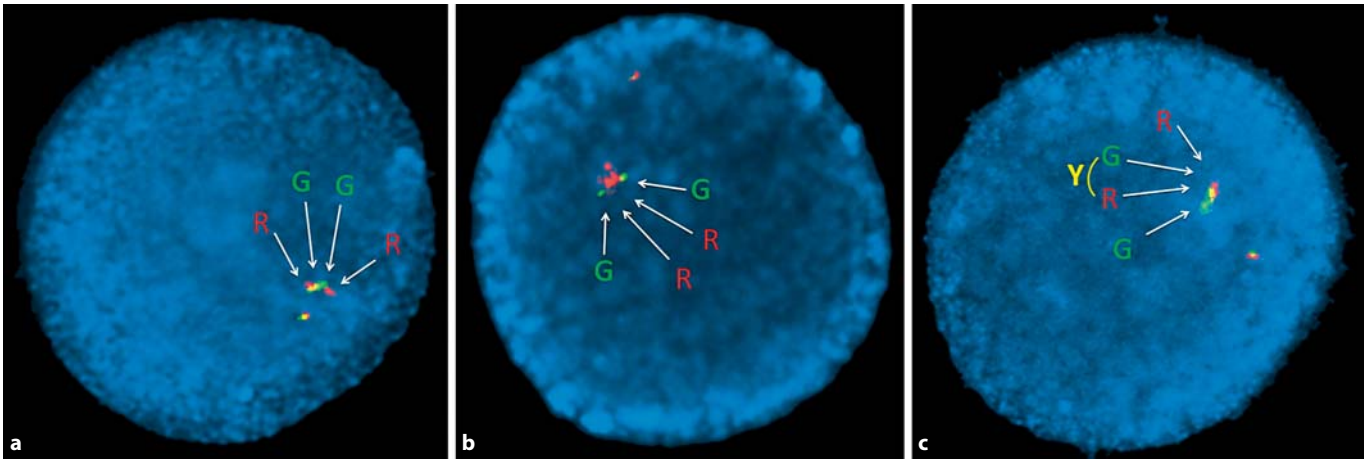

Fig. 2. Fine-scale visualization of the 479-kb duplication detected in 2 individuals that looked identical by aCGH identified substructures of both direct and inverted orientation. **a** 5′ and 3′ probes designed to the ends of the 479-kb duplication at 15q13.3 (29,818,104–30,297,359) covering 23 kb and 56 kb, respectively, labeled in green (5′) and red/orange (3′) show a distinctive signal pattern red-green–green-red (arrows; RGGR) in interphase cells, indicating the presence of an inverted duplication. **b** In dye-swap experiments, 5′ red/orange and 3′ green probes produced the opposite signal pattern, GRRG (arrows), confirming the inversion. The small red and green adjacent signals (without arrows) represent the same region on the normal (unduplicated) chromosome in each cell. **c** The same probes for the 479-kb duplication (5′ green, 3′ red) show distinctly different patterns in the second individual, indicating the presence of a direct duplication (arrows; GRGR). The center region is represented as being yellow (Y) due to the overlap of green and red signals, unlike that observed in **a** and **b**. The small red and green adjacent signals (without arrows) represent the same region on the normal (unduplicated) chromosome.

Resolving the Structure of Duplications

We further investigated the utility of oligonucleotide-based FISH probes for resolving complex genomic structures with a 2-color probe set, designed as described in Materials and Methods. When 2 samples with the identical 479-kb aCGH duplication calls were analyzed by the 2-color probe set, we saw 2 distinctly different staining patterns. One sample revealed characteristic staining of the region in red-green-green-red indicative of a possible inverted duplication (fig. 2a, b), while the other sample showed a staining pattern of green-red-green-red (fig. 2c), suggesting the presence of a direct duplication. The center portion of the likely direct duplication showed yellow co-staining of the red and green signal, unlike that observed in the first individual.

When the dyes on the 5′ and 3′ probes were swapped, consistent signal patterns in the opposite color scheme were observed for both samples (representative images are shown in fig. 2). In all cases, at least 25 interphase cells were analyzed in detail and the same pattern was observed. When we examined signal intensities from the original aCGH experiments to estimate the absolute copy number in this region, both samples appear to have a 1-copy gain of this locus (approximately 30% signal intensity gain), consistent with the FISH analysis.

Discussion

We have demonstrated that the use of de novo synthesized oligonucleotide libraries is a powerful alternative to clone-based methods for generating high performance FISH probes. Routine visualization of very small genomic regions was accomplished with simple experimental procedures, highlighting the ease of use when compared to other methods, which require probe signal amplification for regions of similar size [Raap et al., 1995]. The flexibility afforded by de novo oligonucleotide synthesis provides an opportunity to readily incorporate genomic knowledge into FISH probe design. Our demonstration that careful and strategic in silico selection of each sequence that comprises the probe mixture leads to higher performing FISH probes paves the way for updating FISH as a more valuable tool in molecular cytogenetics.

As demonstrated with 2q13, where our probes targeted a single gene *(NPHP1)*, our method is readily applicable to gene-specific FISH. In homozygous form, deletions of the *NPHP1* gene have been associated with nephronophthisis and in a subset of patients with Joubert syndrome [Parisi et al., 2004; Castori et al., 2005; Doherty, 2009]. Despite its clinical significance, the region where this gene resides is repeat-rich and FISH analysis had pre-

viously been very difficult. Because we are able to design our probes such that no repetitive elements are included, we successfully achieved high signal to noise over the *NPHP1* locus.

All of the loci <125 kb in this study were chosen for analysis because they were not previously visible using traditional FISH methods. Many of these loci are in telomeric and extremely repeat-rich regions (online suppl. table 1). In all but 1 case (17/18; 94% success rate), we were able to obtain reproducible, robust fluorescent signals at these difficult genomic regions. Our control region of 479 kb also showed robust staining without any compromise in signal quality, indicating that there is no upper limit in size range that may render our method specific to smaller regions.

We hypothesize that the main contributing factor for the clean, strong signals obtained by our probes is the result of strategically designing our probes to exclude noise-generating repetitive elements. Although T_m matching the probes also biased the probes to generate higher signal, especially over the smallest regions, both simple tiling and selective tiling generated high performance probes over regions in which clone-based FISH probes had difficulty. Because our probes contain overlapping sequences, it is possible that signal amplification is happening via a network of probes; however, we do not believe that this effect alone can contribute to the improved performance over clone-based FISH, as our initial exploration of end-to-end tiled probes without overlapping sequences also generated a satisfactory signal over several different regions tested (data not shown). The overlapping sequences were added in order to maximize the chances that the probe would find its complementary target DNA, despite variability in the completeness of the denaturation process or loss of specific fragments of DNA from the target chromosome.

Target chromosome variability may account for the variable performance seen by our smallest probe over Xq27.1. Despite there being a difference in performance, the total tiled region sizes for 4p16.3 and Xq27.1 were nearly identical (6.5 kb and 6.8 kb, respectively) with a very similar number of oligonucleotide sequences selected for the probe mixture (64 and 67 oligonucleotides, respectively; online suppl. table 1). However, while the 4p16.3 probe was distributed over a 22-kb region, the Xq27.1 probe was contained within a 7.8-kb region. We speculate that the sample-to-sample variability observed with the Xq27.1 probe may stem from the difficulty in preserving a region as small as 7.8 kb for successful FISH hybridization. This region is also very high in repetitive elements (75% of 31 kb; highest of all 18 loci tested), which may contribute to this region being more susceptible to target DNA variability, as we were able to obtain reproducible success with the 6p22.2 probe, a mere 6.7 kb in size.

The high resolution afforded by the FISH method reported here enabled us to discover 2 different genomic structures in individuals who appeared to have the same duplication by aCGH. Because aCGH is not capable of differentiating the orientation of duplications or detecting inversions, results from aCGH alone suggest that these 2 individuals have the same duplication (online suppl. fig. 2a). The 2-color FISH method provided us with an indication that these 2 individuals have different genomic abnormalities, which we hypothesize to be a difference in the orientation of the duplicons. Our findings demonstrate that high-resolution FISH can quickly and robustly provide independent and complementary information that aids in providing a more complete picture of chromosomal structures than aCGH alone.

Interestingly, this duplicated region overlaps with a reported 15q13.3 microdeletion syndrome associated with mental retardation and seizures, and includes a candidate gene for epilepsy *(CHRNA7)*; specifically, it was reported that the common region between this duplication and the 15q13.3 microdeletion frequently undergoes inversions [Sharp et al., 2008]. There appears to be phenotypic variation in patients with the duplication, ranging from no pathology to some degree of psychiatric disease reported [van Bon et al., 2009]. A recent study has further characterized this region in a large cohort of patients and shown that there are a series of complex sets of low-copy repeats surrounding various breakpoints associated with small and large duplications at this site [Szafranski et al., 2010]. It is still not clear whether duplications similar to what we have seen are clinically relevant, but Szafranski and colleagues have proposed that duplications involving *CHRNA7* may be a risk factor for neurobehavioral disorders.

In addition, we found that a known copy number polymorphism region was duplicated on 2 independent chromosomes, where additional copies associated with 6p25.3 were found on chromosome 16. FISH is one of the best methods to gain information on where additional copies of duplicated sequences are in the genome, and this study demonstrates the necessity to follow-up on aCGH findings of duplications to confirm the physical location of additional copies. As genome-wide association studies of copy number variants are being conducted, it is critical that duplications have FISH validation to determine the

genomic structure and physical location of the duplicated copy.

With the improvements in resolution that cytogenetics is experiencing through the introduction of aCGH, it is critical that complementary molecular methods become available to provide positional and orientation information of chromosome structures from individual cells at similar resolution. The use of complex oligonucleotide libraries as the starting material for FISH probes produced highly reproducible and robust signals in the size ranges that were traditionally difficult to visualize routinely with clone-based FISH. The inherent flexibility of de novo synthesized oligonucleotide libraries is a powerful advantage that can help enable FISH to play a more prominent role in molecular cytogenetics. In addition to the improvements in resolution, our method is highly applicable to the use of FISH on model organisms and other species for which sequence data may be readily available, e.g. from next-generation sequencing technologies, but for which a lack of clone-based resources limit the generation of traditional FISH probes. Through rigorous demonstration of the performance and utility of these oligonucleotide-based, in silico selected probes, we have established that FISH can continue to play a significant role in the study of chromosomes as the field of molecular cytogenetics advances.

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