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Frequency of close positioning of chromosomal loci detected by FRET correlates with their participation in carcinogenic rearrangements in human cells

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Abstract

It has been well established that genes participating in oncogenic rearrangements are non-randomly positioned and frequently close to each other in human cell nuclei. However the actual distance between these fusion partners has never been determined. The phenomenon of Fluorescence Resonance Energy Transfer (FRET) is observed when a donor fluorophore is close (<10 nm) to transfer some of its energy to an acceptor fluorophore. The aim of this study was to validate the use of FRET on directly labeled DNA molecules to assess the frequency of positioning at <10 nm distances between genes known to be involved in rearrangement and to correlate it with their probability to undergo rearrangement. In the validation experiments, the frequency of FRET-SE (sensitized emission) was found to be 93-96% between probes for the immediately adjacent chromosomal regions as compared to 0.1-0.2% between probes for the random loci located on large linear separation. Further, we found that the frequency of FRET-SE between four pairs of genes that form rearrangements in thyroid cancer was 5% for *RET* and *CCDC6*, 4% for *RET* and *NCOA4*, 2% for *BRAF* and *AKAP9*, and 2% for *NTRK1* and *TPR*. Moreover, the frequency with which FRET was observed showed strong correlation ($r = 0.9871$) with the prevalence of respective rearrangements in thyroid cancer. Our findings demonstrate that FRET can be used as a technique to analyze proximity between specific DNA regions and that the frequency of gene positioning at distances allowing FRET correlates with their probability to undergo chromosomal rearrangements.

Keywords

Chromosomal rearrangement; FRET; rearrangement prevalence

INTRODUCTION

Recurrent chromosomal rearrangements are commonly found in human cancers such as leukemia, lymphoma, thyroid cancer and prostate cancer. Since the proximity between recombinogenic loci was first demonstrated a decade ago (Nikiforova et al., 2000), there has been an ever-increasing body of evidence showing that genes that participate in chromosomal rearrangements are positioned non-randomly and close to each other in nuclei of interphase human cells. Using fluorescence in situ hybridization (FISH), proximity

between rearrangement-prone loci has been shown for genes involved in intrachromosomal rearrangements in thyroid cells, such as *RET* and *CCDC6* (Nikiforova et al., 2000), *RET* and *NCOA4* (Gandhi et al., 2006), and *NTRK1* and *TPR* (Roccato et al., 2005), intrachromosomal rearrangements in prostate cells such as *TMPRSS2* and *ERG* and *TMPRSS2* and *ETV1* (Lin et al., 2009; Mani et al., 2009), and interchromosomal rearrangements in lymphomas such as *MYC*, *BCL* and immunoglobulin genes (Roix et al., 2003). Spatial proximity between recombinogenic genes in these studies was observed as juxtaposition of FISH probes seen by confocal microscopy, although a more precise estimation of distances between the fusion gene partners could not be provided. This is because standard confocal microscopy does not allow a xy-resolution smaller than 250 nm.

Recent advances in fluorescent microscopy offer additional tools that can be used to resolve much smaller distances between probes. Specifically, fluorescence resonance energy transfer (FRET) is observed when a donor fluorophore is close enough to transfer some of its energy to an acceptor fluorophore (Lakowicz, 1983). For FRET to occur, (1) the donor molecule should be fluorescent with a sufficiently long lifetime, (2) the donor emission spectrum should overlap with the acceptor excitation spectrum, and (3) the donor and acceptor molecule should be in close proximity to each other (<10 nm) (Gordon et al., 1998). The probability for energy transfer can typically be assessed via FRET-SE (sensitized emission) and FRET-AB (acceptor photobleaching) techniques (Wouters et al., 2001).

Common applications of FRET have included fluorescently labeled DNA oligomers in real-time PCR, cAMP or calcium binding assays, and studies involving protein folding and protein-protein interactions after immunostaining (Kenworthy, 2001; Grecco and Verveer, 2011). FRET has also been used to study DNA-protein interactions (Matsumoto et al., 2009), detection of mRNA splice variants after PNA-FISH (Robertson et al., 2006; Blanco and Artero, 2010), and in combination with single-molecule techniques to study exonuclease degradation of DNA constructs (Lee et al., 2011).

In this study we explored the possibility to use FRET for estimating distances between two DNA regions. As a model system, we utilized human thyroid cells which give rise to tumors that harbor several different types of chromosomal rearrangements. The most common chromosomal rearrangement in papillary thyroid cancer is *RET/PTC*, which involves the *RET* gene located on 10q11.2 to different partner genes. The most prevalent *RET/PTC* types are *RET/PTC1*, which is a fusion between *RET* and *CCDC6* (10q21) (Grieco et al., 1990), and *RET/PTC3*, which is a fusion between *RET* and *NCOA4* (10q11.2) (Bongarzone et al., 1994; Santoro et al., 1994). Other types of chromosomal rearrangements known to occur in papillary thyroid cancer involve the *NTRK1* gene, such as *NTRK1-TPM3* inv(1)(1q21-q21.2) (Butti et al., 1995) and *NTRK1-TPR* inv(1)(1q21-q25) (Greco et al., 1992), and the *BRAF-AKAP9* rearrangement (inv(7)(q21-22q34)) (Ciampi et al., 2005). In sporadic tumors, these rearrangements occur with significantly different frequencies (Musholt et al., 2000; Nikiforov, 2002; Ciampi et al., 2005; Brzezianska et al., 2006; Lee et al., 2006; Greco et al., 2010).

In this study, we validate the use of FRET on directly labeled DNA molecules to assess the frequency of positioning at <10 nm distances between specific gene pairs and explored the relationship between frequency of location at distances allowing FRET and gene involvement in fusions found in thyroid cancer.

MATERIALS AND METHODS

Tissue preparation and fixation

Normal human thyroid tissues were harvested immediately after surgery for non-malignant thyroid disease using the University of Pittsburgh Internal Review Board approved protocol and used to obtain touch preparations of human thyroid cells. Cells were fixed following a 3D fixation protocol to preserve their native chromatin architecture as previously described (Solovei et al., 2002a; Solovei et al., 2002b). Briefly, touch preparation on positively charged slides were fixed in 3.7% PFA followed by treatment with 0.5% TritonX-100 and incubation in 20% glycerol. Slides were subjected to four rounds of free-thaw treatment in liquid nitrogen followed by washes in PBS, 0.1M HCl and 2XSSC, and stored in 50% formamide/2X SSC at 4°C until use. Care was taken to prevent air drying at any point in the preparation process.

DNA Probes and FISH

BAC clones (100-150 Kb size) RP11-351D16 (*RET*), RP11-481A12 (*NCOA4*), RP11-435G3, RP11-369L1 (*CCDC6*), RP11-107D16 (*NTRK1*), RP11-295K2 (*TPR*), RP11-726N20, RP11-839B19 (*BRAF*), and RP11-441O15 (*CNNM1*) were obtained from BAC/PAC Resources, Children's Hospital, Oakland. Purified BAC probes were extracted using the Plasmid Midi Kit (Qiagen) according to the manufacturer's protocol. BAC clone RP11-481A12 containing the human *NCOA4* gene was subcloned into fosmid vector after cutting with restriction enzymes (Epicentre). A mixture of subcloned probes (SC10, SC19) containing 70 kb of the *NCOA4* gene and its flanking regions was used as a probe for *NCOA4* (Gandhi et al., 2006). 1.5µg of BAC DNA was fluorescently labeled by nick translation with 0.02 mM of Alexa 488-dUTP or Alexa 594-dUTP (Invitrogen) to yield 200-500 bp fragments of labeled DNA. Labeled probes were co-precipitated with human Cot-1 DNA and salmon sperm DNA and resuspended in hybridization buffer to obtain the final probe for FISH. Slides containing 3D-fixed nuclei (described earlier) were treated with Collagenase H, washed in PBS and post-fixed in 10% formalin. Fluorescently labeled probe was applied and the slides were hybridized overnight at 37°C. Post-hybridization washes were carried out in 50% formamide/2X SSC and 2X SSC and slides were counterstained in fluorescent mounting media without DAPI.

Sensitized emission FRET microscopy

Nuclei were imaged on a Leica SP5 TCS 4D confocal laser scanning fluorescence microscope using a 63x, 1.4 N.A. oil PlanApo objective. We used the FRET-SE wizard of the Leica Application Suite Advanced Fluorescence (LAS AF) interface for the FRET analysis. Donor (D) and acceptor (A) fluorescence signals were detected following excitation with visible Argon laser and HeNe 594 laser respectively. Image stacks were acquired in a 512 × 512 format, 2 line averages, 6-7× zoom and 0.13 µm step size resulting in voxel dimensions of approximately 74 nm × 74 nm × 130 nm. The Alexa 488 and Alexa 594 fluorescence signals were detected through 495-565 nm and 600-750 nm band-pass filters of the scanning unit using a 488/594 dichroic and gains set to 1100. The D-A fluorophores have been previously used for FRET-SE experiments (Mukhopadhyay et al., 2007). The choice of D-A combination was determined by the experimental design that utilized 100-150 kb BAC probes. The intention was to have sufficient spectral overlap to allow for FRET while minimizing spectral bleed through. Donor only (Alexa488) and acceptor only (Alexa594) references were included to obtain calibration coefficients to correct for the D-A excitation and emission crosstalk, and for the impact of background signal. Since it was not possible to determine the concentration of donor and acceptor fluorophores after labeling of the FISH probes, the most conservative approach for FRET-SE was followed which utilized three filter sets and calculating the corrected measure of

FRET by the formula $E_A(i) = \frac{B - A \times \beta - C \times (\gamma - \alpha \times \beta)}{C \times (1 - \beta \times \delta)}$, where A, B, C correspond to the intensities of the 3 signals (donor, FRET, acceptor) and α , β , γ and δ are calibration factors generated by acceptor and donor only references (van Rheenen et al., 2004). This method is least likely to produce a false positive result. However, the drawback is that this method is less sensitive than other FRET measurement methods and results in lower FRET efficiency values (Gordon et al., 1998). All calculations were performed on background-subtracted images. FRET-SE analysis was carried out in 3D by drawing a circular region of interest (ROI) of at least 25 pixels where colocalization of donor and acceptor signals was observed. On average, 500 nuclei from each of two different donors were scanned and analyzed for (a) proportion of signals that exhibited FRET (FRET frequency) and (b) FRET efficiencies (Eapp).

Statistical analysis

Comparison of FRET between probe pairs was performed using ANOVA with Tukey-Kramer correction for determining whether the means are significantly different from one another. Pearson's product-moment correlation coefficient (r) was calculated by plotting FRET frequency on the X-axis and genomic distance and rearrangement prevalence on the Y-axes. The difference between two values was considered significant when the P value was less than 0.05.

RESULTS

Validation of FRET for evaluating nanometer distances between DNA regions in human cells

To establish whether FRET could be used for assessing extremely small (< 10 nm) distances between fluorescently tagged intranuclear DNA targets, we first performed experiments on human thyroid TPC-1 cells. This cell line harbors *RET/PTC1* rearrangement that is formed by the genomic fusion of the *RET* and *CCDC6* genes, and hence offers a control for genes positioned immediately next to each other on the DNA molecule. FRET-SE analysis revealed energy transfer in 100 out of 105 (95.24%) nuclei (Fig. 1). The FRET efficiencies (Eapp) ranged from 5% to 26% with the mean Eapp of 13.5% (Fig. 1c). To determine whether the phenomenon of energy transfer was not dependent on the choice of fluorescent labels used for specific probes, the analysis was repeated after fluorophore switching. It showed similar frequency of FRET occurrence (96.15%) and similar Eapp values (range, 5% to 25%; mean, 13.1%) (Fig. 1d). To assess the specificity of FRET, we assessed the occurrence of FRET between chromosomal loci that are located on separate chromosomes and do not participate in fusion. For this, TPC-1 cells were co-hybridized with probes for *RET* and *BRAF*. Visual analysis of 1000 nuclei showed that *RET* and *BRAF* probes were positioned at variable distances far away from each other and were colocalized in less than 1% of the analyzed nuclei. Of those, only 1 pair of signals exhibited FRET (Eapp = 11%), resulting in a 0.1% frequency of FRET between *RET* and *BRAF* probes. This demonstrates an extremely low frequency of FRET detected between random chromosome loci.

Next, we explored if this approach can be used in primary human thyroid cells instead of a cell line. To assess the FRET efficiency between closely spaced chromosomal loci, we used two BAC probes for adjacent regions of the *BRAF* gene (RP4-726N20 and RP4-839B19). FRET analysis showed energy transfer in 96.2% of random signal pair with Eapp values ranging from 5% to 28% (mean, 13.7%). As a negative control, we used probes for *RET* (10q11.2) and *CNNM1* (10q24.2), two loci that are located on 57 Mb distance from each other on 10q. In 1000 nuclei that were analyzed, only 2 signals (0.2%) demonstrated FRET (Eapp = 7% and 8%).

These experiments allowed us to establish that FRET-SE can be used in a setting of conventional DNA FISH and that FRET can be consistently registered between DNA probes spatially located next to each other, in contrast to a very rare occurrence of FRET between random DNA loci.

Rearrangement-prone DNA loci demonstrate FRET and therefore can be located at nanometer distances from each other

Next, we used the validated approach, i.e. FRET in combination with DNA-FISH, to assess the mutual location of four pairs of genes involved in recurrent carcinogenic chromosomal rearrangements in human thyroid cancer cells: *RET* and *CCDC6*, *RET* and *NCOA4*, *BRAF* and *AKAP9*, and *NTRK1* and *TPR* (Fig. 2). In all of these gene pairs, the partners are located on the same chromosome, but on different genomic separation that ranges from 8Mb to 49Mb. The frequency of FRET was $5.31 \pm 0.66\%$ between *RET* and *CCDC6*; $3.57 \pm 0.58\%$ between *RET* and *NCOA4*; $1.77 \pm 0.41\%$ between *BRAF* and *AKAP9*; and, *NTRK1* and *TPR* was $2.07 \pm 0.45\%$ (Fig. 2d). The frequency of FRET found in all four pairs was significantly higher than the background level of 0.20% found in the same experiment between probes located on chromosome 10q and not involved in rearrangement, *RET* and *CNNM1* ($p < 0.005$). Of the four gene pairs studied, the frequency of FRET was significantly higher for *RET* - *CCDC6* as compared with *BRAF* - *AKAP9* and *NTRK1* - *TPR* (ANOVA $p < 0.05$). The mean Eapp value was 6% for *RET* - *CCDC6*, 8% for *RET* - *NCOA4*, and 10% for *BRAF* - *AKAP9* and *NTRK1* - *TPR*.

These results indicate that genes that participate in chromosomal rearrangements can be positioned at a distance less than 10 nm from each other in a distinct portion of normal human cells, thereby allowing energy transfer to occur between the corresponding probes.

Frequency of gene pair location permitting FRET correlates with the prevalence of corresponding gene rearrangement in human tumors

Since all four pairs of rearrangements studied are involved in intrachromosomal rearrangements, it is conceivable that the frequency of FRET simply correlates with the linear separation between the probes, and this in turn correlates with the frequency of recombination between specific gene pairs. Our analysis showed that frequency of FRET observed between the four studied gene pairs did not correlate with genomic distance ($r = 0.7093$, $p = 0.291$), but demonstrated strong positive correlation with the prevalence of specific gene rearrangement types in thyroid cancer ($r = 0.9871$, $p = 0.013$) (Fig. 3).

This suggests that spatial positioning of specific chromosomal loci with respect to each other does not follow genomic separation between the loci located on the same chromosome, but the frequency of close spatial proximity between specific loci correlates with their probability to participate in gene fusion in human cancer cells.

DISCUSSION

The results of this study demonstrate that FRET can be successfully combined with FISH and used as a technique to identify DNA regions located very close, i.e. at nanometer distances from each other. Use of this method provides novel information on the spatial relationship between DNA loci and their probability to undergo carcinogenic chromosomal rearrangements in human cells.

Light microscopy techniques including standard confocal microscopy allows a xy-resolution of 250 nm. Any two objects located at a distance closer than 250 nm appear colocalized. In standard cytogenetic interpretation, two FISH signals are considered juxtaposed when the distance between the two probes is no larger than the size of the signal. In this study, for the

first time, we provide estimation of distance between colocalized probes by applying the principle of energy transfer. FRET occurs when two molecules are located sufficiently close to each other, generally less than 10 nanometers. FRET has been applied to study protein-protein interactions, DNA-protein interactions, fluorescently tagged DNA oligomers in conjunction with real-time PCR and single molecule analysis and even RNA-FISH to study alternative splicing variants (Kenworthy, 2001; Robertson et al., 2006; Mukhopadhyay et al., 2007; Matsumoto et al., 2009; Blanco and Artero, 2010; Grecco and Verveer, 2011; Lee et al., 2011). However, prior to this study, this technique has not been used to study DNA-DNA interaction in combination with conventional FISH.

In this study, the range of FRET efficiencies observed between the D-A pairs ranged from 5% to 28%. For the D-A pair, Eapp values less than 5% were excluded from the analysis since it represented a Försters distance greater than 10 nm, beyond which energy transfer typically does not occur. The relatively low level of FRET efficiencies observed could either be a true representation of the spatial separation between the genes that we analyzed, or may reflect the choice of donor-acceptor fluorophores and the fact that these experiments were conducted in fixed cells. Low levels of FRET (mean Eapp – 5%-15%) have been reported before (Fjorback et al., 2009). In fact, findings have been concluded based on fluctuations between low and high FRET efficiencies (Mukhopadhyay et al., 2007; Lee et al., 2011). Another explanation for observing low levels of FRET efficiencies could be the fact that we used the most conservative method of calculating Eapp values (van Rheenen et al., 2004). Although this method yields low Eapp values, the advantage of this technique is that it provides clean background FRET intensity and reduces falsely positive readings. In fact, when the same ROIs were evaluated by the other two methods of calculating FRET efficiencies (ratiometric, and as described by Wouters et al (Wouters et al., 2001), higher Eapp values were observed. Since we used BAC probes that were between 100-150 kb in length instead of nucleotide oligomers, and the fact that it was not possible to quantify the amount of fluorophore incorporated, we elected to use a D-A combination that did not have a significant crosstalk and compounded it with a conservative technique of Eapp calculation. Although we realize that this approach may underestimate the frequency of FRET, it has a very low chance of false positive FRET detection.

It is well known that exposure to ionizing radiation causes double-strand breaks (DSB) in the DNA resulting in the formation of carcinogenic chromosomal rearrangements. The debate between the breakage first hypothesis and the contact first hypothesis is still ongoing (Savage, 2000). The breakage first hypothesis suggests that after DSB, free DNA ends migrate through the nuclear space and somehow finds its fusion partner (Lea, 1946). Time-lapse monitoring of γ H2AX foci after DNA damage have shown that the free DNA ends are indeed mobile and that this mobility can occur up to several microns in distance (Aten et al., 2004). However, counter arguments to this have also been presented that show the positional stability and limited mobility of radiation-induced DSB, thereby having no effect on the increased probability of translocations (Jakob et al., 2009). The contact-first hypothesis, on the other hand, argues that certain genomic regions are positioned close to each other prior to DNA damage, so that free DNA ends are easily amenable to undergo rearrangement in the neighboring space (Sachs et al., 1997). This hypothesis has been supported by multiple studies demonstrating that potential recombinogenic loci are positioned in proximity to each other in the nucleus of normal human cells (Nikiforova et al., 2000; Roix et al., 2003; Roccato et al., 2005; Gandhi et al., 2006; Mani et al., 2009). Many of these rearrangements involve genes on the same chromosome, suggesting that this may be a factor determining the rearrangement frequency. However, the results of this study suggest that spatial location at very close distance rather than residing on the same chromosome arm, correlates with the frequency of rearrangements found in cancer.

In summary, this study demonstrates for the first time the use of FRET in combination with DNA-FISH to estimate distances between closely spaced DNA regions and show that potential recombinogenic genes can be positioned in human interphase nuclei within nanometer distances of each other, and that the frequency of such close proximity correlates strongly with their probability to undergo chromosomal rearrangements, thereby providing support to the contact-first hypothesis for the formation of chromosomal rearrangements in human cells.

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Figure 1.

FRET between DNA-FISH probes in TPC-1 human thyroid cancer cells known to harbor *RET/PTC1* rearrangement and have chromosome 10 trisomy. **(a)** 2D projection image of the nucleus showing three *RET* (Alexa 594) and three *CCDC6* (Alexa 488) signals with overlap of one pair of *RET* and *CCDC6* (arrow). Scale, 2 μm . **(b)** Confocal images of the region of interest (ROI) that encompasses the fused *RET* and *CCDC6* loci seen in (a) that register the presence of FRET in the ROI. Scale, 1 μm . **(c)** Stack profile charts for intensities observed in Donor, FRET, Acceptor channels and Eapp values in the ROI with Donor (*CCDC6*) and Acceptor (*RET*) colocalization. FRET-SE is observed only the z-planes where the two probes are colocalized whereas the background Eapp values are zero. Maximum Eapp value is observed in the same z-plane in which peak FRET-SE intensity is observed. **(d)** Mean Eapp values observed in TPC-1 cells between *RET* (Alexa488) and *CCDC6* (Alexa 594) (grey bars) and after fluorophore switching, *RET* (Alexa 594) and *CCDC6* (Alexa488) (black bars) showing similar frequency distributions ($n = 100$ nuclei for each experiment).

Figure 2. FRET-SE for DNA-FISH probes in primary human thyroid cells. **(a)** 2D projection of the nucleus hybridized with the *RET* (Alexa 594) and *CCDC6* (Alexa488) probes. One pair of probe shows colocalization (arrow). Scale – 1 μm. **(b)** Mean FRET frequencies for *RET-CCDC6*, *RET-NCOA4*, *BRAF-AKAP9*, *NTRK1-TPR* and *RET-CNNM1* (negative control) in primary human thyroid cells. Error bars represent S.E.M (n=500, 2 donors).

Figure 3.

Correlation analysis of FRET frequency with genomic distance (blue) and rearrangement prevalence (red) for *RET-CCDC6*, *RET-NCOA4*, *BRAF-AKAP9*, and *NTRK1-TPR*. The correlation coefficients (r) for each analysis are specified. Significant correlation between FRET frequency and rearrangement prevalence ($p = 0.013$) is denoted in bold.

Table 1

Genomic separation of probe pairs and prevalence of the respective rearrangements in human thyroid tumors

Probe pair	<i>RET-NCOA4</i>	<i>RET-CCDC6</i>	<i>BRAF-AKAP9</i>	<i>NTRK1-TPR</i>
Genomic Separation (Mb)	8	18	49	29
Prevalence in sporadic PTC (%)[*]	3.9	9.9	0.5	1.3

^{*} based on the prevalence reported by Musholt, et al. 2000; Nikiforov 2002; Ciampi, et al. 2005; Brzezianska, et al. 2006; Lee, et al. 2006. The prevalence of each rearrangement was calculated as the overall prevalence in sporadic papillary thyroid cancer (PTC).