Original Article

Strand-specific fluorescence in situ hybridization: the CO-FISH family

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Abstract. The ability to prepare single-stranded chromosomal target DNA allows innovative uses of FISH technology for studies of chromosome organization. Standard FISH methodologies require functionally single-stranded DNAs in order to facilitate hybridization between the probe and the complementary chromosomal target sequence. This usually involves denaturation of double-stranded probes to induce temporary separation of the DNA strands. Strand-specific FISH (CO-FISH; Chromosome Orientation-FISH) involves selective removal of newly replicated strands from DNA of metaphase chromosomes which results in single-stranded target DNA. When single-stranded probes are then hybridized to such targets, the resulting strand-specific hybridization is capable of revealing a level of information previously unattainable at the cytogenetic level. Mammalian telomeric DNA consists of tandem repeats of the (TTAGGG) sequence, oriented $5' \rightarrow 3'$ towards the termini of all vertebrate chromosomes. Based on this conserved structural organization, CO-FISH with a telomere probe reveals the absolute $5' \rightarrow 3'$ orientation of DNA sequences with respect to the pter $\rightarrow$ qter direction of chromosomes. Development and various applications of CO-FISH will be discussed: detection of cryptic inversions, discrimination between telomeres produced by leading- versus lagging-strand synthesis, and replication timing of mammalian telomeres.

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CO-FISH

CO-FISH is elegant in its simplicity (Fig. 1). It requires cultivation of cells in the presence of bromodeoxyuridine (BrdU) and/or bromodeoxycytidine (BrdC) for a single round of replication (a single S phase). Due to the semiconservative nature of DNA synthesis, each newly replicated double helix contains one parental DNA strand plus a newly synthesized strand in which the nucleotide analogs have partially replaced thymidine and/or deoxycytidine (unifilar substitution). Following preparation of metaphase chromosomes on microscope slides by standard cytogenetic techniques, exposure of cells to UV light in the presence of the photosensitizing DNA dye Hoechst, results in numerous strand breaks that occur preferentially at the sites of BrdU incorporation. Nicks produced in the chromosomal DNA by this treatment then serve as selective substrates for enzymatic digestion by Exo III. This results in the specific removal of the newly replicated strands while leaving the original (parental) strands largely intact. Thus, for the purposes of subsequent hybridization reactions, the two sister chromatids of a chromosome are rendered single stranded, and complementary to one another, without the need for thermal denaturation. The unressected parental strands then serve as single-stranded target DNA for hybridization with single-stranded probes. The approach has proven to be quite robust, limited only by the present paucity of suitable single-stranded probes.

"Humble beginnings"

Chromosome-Orientation Fluorescence in Situ Hybridization (CO-FISH) was first described in 1993 as a method for making in situ hybridization strand specific (Goodwin and Meyne, 1993; Goodwin et al., 1993). The technique demonstrated that the repetitive alphoid DNA sequences in the centromeric region of human chromosome 18 exist as head-to-tail

tandem arrays. The technique was named for its ability to determine the orientation of tandem repeats, relative to each other, along DNA strands within chromosomes.

Lateral asymmetry refers to the unequal fluorescent intensity between adjacent regions of sister chromatids observed in the centromeric regions of mitotic chromosomes of mouse or human origin when cells are grown in BrdU for a single round of DNA synthesis (Lin et al., 1974a, b). In an early application, CO-FISH was used to show that mouse pericentromeric satellite DNA (satDNA) was also oriented head-to-tail and the regions of asymmetrical brightness coincided with mouse major satellite repetitive DNA. Importantly, the more heavily BrdU-substituted chromatid was the one that fluoresced less brightly (Goodwin et al., 1996). In this instance, the strand specificity of CO-FISH provided support for a 20-year-old hypothesis on the origin of lateral asymmetry.

In species with telocentric or acrocentric chromosomes, a common structural anomaly referred to as a Robertsonian (Rb) translocation occurs when two of these V-shaped chromosomes fuse at their centromeres to form a single X-shaped metacentric chromosome. Analysis of the molecular composition and organization of the pericentromeric regions of Rb translocations between mouse telocentrics demonstrated the absence of pericentromeric telomeric sequences near the fusion points of Rb chromosomes, but retention of detectable amounts of minor satellite DNA (Garagna et al., 1995). The CO-FISH technique was used to further analyze both the major and minor pericentromeric satDNAs of telocentrics and Rb chromosomes. To detect the very small amount of minor satDNA, a sensitive modification was developed that combined CO-FISH with primed in situ labeling and conventional FISH. It was found that both the major and the minor satDNA tandem repeats are oriented head-to-tail in telocentric and Rb chromosomes, and their polarity is always the same relative to the centromere (Garagna et al., 2001).

"And then there were – telomeres"

Telomeric DNA consists of tandem arrays of short, repetitive G-rich sequence (TTAGGG in vertebrates) that are oriented $5' \rightarrow 3'$ towards the end of chromosomes (Meyne et al., 1989; Blackburn, 1991) and form a $3'$ single-stranded G-rich overhang (Makarov et al., 1997; Wellinger and Sen, 1997). The C-rich telomere strand is at the $5'$ end and the G-rich telomere is at the $3'$ end of each chromosomal DNA strand, identifying the absolute $5' \rightarrow 3'$ pter $\rightarrow$ qter directionality of the DNA helical strand. When the strand specificity of CO-FISH was combined with this knowledge of the structural organization of eukaryotic telomeres, we were able to demonstrate that the procedure could be used to determine the absolute direction of various repetitive sequences anywhere along the DNA helix within chromatids (Meyne and Goodwin, 1995). This variation of the CO-FISH method is sometimes referred to as "COD-FISH" (Chromosome-Orientation and Direction FISH) to indicate this fact. Simply put, any two DNA sequences share the same relative orientation if their respective CO-FISH signals appear on the same sister chromatid. If, in addition, the absolute orientation is known for one (e.g., telomeres), then the absolute $5' \rightarrow 3'$ pter $\rightarrow$ qter is also known for the other.
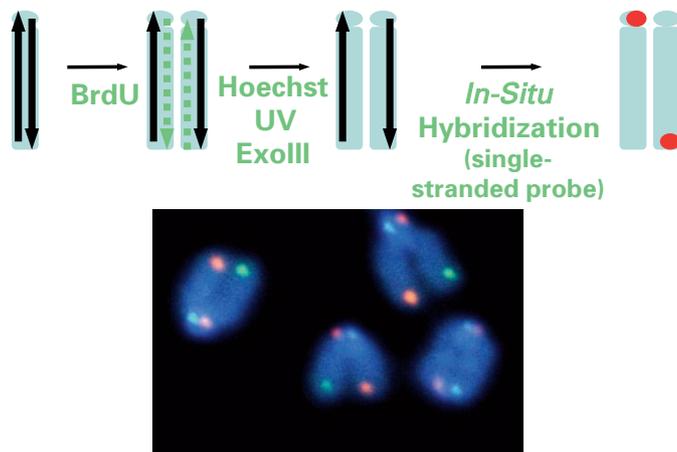
Fig. 1. Chromosome-Orientation FISH (CO-FISH). Mitotic cells are collected after culture in bromo-substituted nucleotides (e.g., BrdU) for a single cell cycle. Fixed cells on microscope slides are stained with the DNA-binding fluorescent dye Hoechst 33258. Exposure to UV light nicks the substituted strand, and exonuclease III digests it. The process effectively removes the newly synthesized DNA strands and leaves behind the two parental strands that are now located on sister chromatids. A single-stranded probe hybridizes to complementary telomeric DNA on one chromatid of each chromosome arm producing a two-signal pattern instead of the four signals seen with ordinary FISH. CO-FISH hybridization with both the G-rich telomere probe (green; identifies the telomere produced via leading-strand DNA synthesis) and the C-rich telomere probe (red; identifies the lagging-strand telomere), illustrating the exquisite strand specificity of the technique.

Inversion detection

Inversions are notoriously difficult to detect by cytogenetic methods. The ability of CO-FISH to restrict hybridization of single-stranded probes to only one of the two sister chromatids (for any unique locus) means that it can also be used for inversion detection. Because an inversion reverses the orientation of DNA sequences within the inverted region, it becomes visible as a "jump" or "switch" in probe signal from one chromatid to its sister. Such a switch is easily detected when compared to a reference probe outside of the inverted region; telomeric sequences are convenient for this purpose. Information provided by such a signal switch is not constrained by the theoretical limitations of light optics. Thus, in principle, the size of the inversion that can be detected is limited only by the robustness of the probe itself. In practice, CO-FISH was first used to detect obligate chromosomal inversions associated with isochromosome formation in two human cell lines (Bailey et al., 1996a). Shortly thereafter, pericentric inversions in chromosomes 1, 8, 10, and X, previously identified by chromosome banding, were confirmed with CO-FISH (Bailey et al., 1996b).

Telomeres produced by leading- and lagging-strand synthesis behave differently

Studies in our laboratory were the first to demonstrate that DNA double-strand break (DSB) repair genes (DNA-PKcs, Ku70 and Ku86) play a dual role in maintaining chromosomal stability in mammalian cells. Besides their more obvious function in repairing incidental DNA damage to the genome, such

repair proteins help to protect the ends of telomeres from degradation and untoward acts of recombination through their "end-capping" function (Bailey et al., 1999). Subsequent CO-FISH studies led to the discovery that there are strand-specific differences in the postreplicative processing of mammalian telomeres (Bailey et al., 2001). Because replication of telomeric DNA begins at internal origins and proceeds towards the chromosomes' ends, CO-FISH hybridization patterns produced with G-rich telomere probes [i.e., labeled $(TTAGGG)_n$ oligomeres] specifically identify telomeres produced by leading-strand synthesis, whereas hybridization with the complementary C-rich probes identify "lagging-strand telomeres". Telomeres produced via leading-strand DNA synthesis were especially (and perhaps exclusively) susceptible to inappropriate fusion under conditions that compromised components of the end-capping machinery. These, for example, included deficiencies in DNA-PKcs and TRF2 (telomere repeat binding factor 2). Under these conditions, chromosomes could be seen to fuse to one another – end-to-end, often in extensive arrays involving several chromosomes – as if the telomeres were mistakenly recognized as DSB ends in desperate need of repair. Each fusion was the result of interaction between telomeres produced by leading-strand synthesis. Such fusions typically retained large blocks of telomeric sequence at the point of joining, so they were not the result of loss or severe erosion of telomeric DNA [an observation that has been confirmed by measuring telomere length through the junctions (Gilley et al., 2001; Goytisolo et al., 2001)]. Banding studies demonstrated no bias towards involvement of specific chromosomes. Several of the fusions were clonal, suggesting they are true covalent DNA linkages produced by recombinational events, rather than merely being the result of telomeric associations of a less permanent nature.

To investigate further the role of DNA repair proteins in mammalian telomere protection, DNA-PK-proficient mouse cells were exposed to a highly specific inhibitor of DNA-PKcs kinase activity (Kashishian et al., 2003) for a single cell cycle. Telomere-positive chromosomal end-to-end fusions were induced in a concentration-dependent manner, demonstrating that the telomere end-protection role of DNA-PK requires its kinase activity (Bailey et al., 2004a). The fusions were uniformly chromatid-type, consistent with a role for DNA-PK in capping telomeres after DNA replication. Importantly, CO-FISH analyses again demonstrated that the fusions preferentially involved telomeres produced via leading-strand DNA synthesis, as we have shown previously (Bailey et al., 2001; Espejel et al., 2002). These studies support a model in which the kinase activity of DNA-PK is crucial to reestablishing a protective terminal structure immediately following replication, specifically on telomeres replicated by leading-strand DNA synthesis.

Telomere-DSB fusions identified

In normal repair-proficient cells, ionizing radiation (IR)-induced exchanges, such as dicentrics, do not contain telomeric sequences at the point of fusion (Cornforth et al., 1989). We imagined, however, that under conditions of impaired end-capping dysfunctional mammalian telomeres might join not only with each other, but also to IR-induced DSB ends. Neither mouse nor human cells ordinarily contain cytogenetically visible blocks of telomeric sequence at internal sites, so the occurrence of interstitial telomeric sequence following IR would indicate that a telomeric fusion has taken place. We devised a strategy to differentiate between telomere-to-telomere and telomere-to-DSB chromosome-type telomere fusion events based simply on the number of internalized telomeric CO-FISH signals – two vs. one, respectively (Bailey et al., 2004b). For cells with DNA-PK-deficient backgrounds, we observed that telomere-DSB rejoining accounted for approximately half of the radiation-induced chromosomal exchanges within the dose range of our experiments. Thus, CO-FISH was instrumental in demonstrating a previously unrecognized pathway for the formation of exchange-type aberrations in cells with dysfunctional telomeres.

Telomeric Sister Chromatid Exchange (T-SCE)

Sister chromatid exchange (SCE) is arguably the largest source of mitotic recombination in mammalian cells. Although conventional cytogenetic analyses have not revealed a terminal bias for SCE formation, high rates of mitotic recombination within sub-telomeric regions have been inferred from CO-FISH studies (Cornforth and Eberle, 2001). More recently we, and others have observed (at least what appears to be) a similar phenomenon in the telomere proper, as evidenced by reciprocal "split" telomere CO-FISH signals (Bailey et al., submitted). These events have been termed T-SCE (Bechter et al., 2004; Londono-Vallejo et al., 2004) and have been implicated as a marker of certain human cancers whose cells are sometimes forced to utilize telomerase-independent pathways to maintain telomere length, the so-called ALT pathway (alternative lengthening of telomeres).

Replication timing during S phase and ReDFISH

A final example of how modifications to the basic CO-FISH approach provides information not easily obtained through other means involves the study of replication timing of particular sequences during S phase. To this point we have considered it a requirement that a cell be unifilarly labeled in each sister chromatid, meaning that it must go through a complete round of DNA synthesis in the presence of halogenated nucleotide precursors. Otherwise, a normal FISH hybridization signal will be produced for each region along the chromosome for which incorporation is incomplete. That is, two isolocus signals will appear on each of the two sister chromatids, rather than the characteristic single-chromatid signal produced by CO-FISH. This means that replication in the presence of BrdU/dC will cause a "switch" from a FISH pattern to a CO-FISH pattern for the locus or sequence under study. One of the two sister chromatids is rendered incapable of providing a target for subsequent hybridization – it becomes "detargeted" – upon passage of the replication fork in the presence of BrdU/dC (note that for the special case of telomeres, the switch is from four FISH signals to two CO-FISH signals). Replicative Detargeting FISH (ReDFISH) takes advantage of this fact by introducing the base analogs at selective times during S phase, and then observing later in mitosis the frequency of CO-FISH signals in comparison to FISH signals. There are a number of ways to achieve this effect. In one such experiment, we added BrdU/dC to a series of

human cell cultures in exponential growth. At regular intervals we then made CO-FISH preparations until all of the cells in S phase at the time of BrdU/dC addition had reached mitosis. Analysis of this (Cornforth et al., 2003) and similar experiments led us to conclude that – quite unlike the situation in yeast, whose telomeres are late replicating – telomeres of mammalian cells replicate throughout S phase (Wright et al., 1999). These two laboratories are presently refining ReDFISH in order to answer other questions related to replication timing, such as whether certain chromosomes replicate their telomeres earlier than others, or whether p- and q-arm telomeres of the same chromosome replicate at the same time (Zou et al., submitted).

Future directions

CO-FISH and related techniques have been put to many uses, some of which were scarcely imagined at the time of its inception. Conversely, it has not found widespread application in areas that we did envision a major impact as, for example, in determining the absolute directional status of repetitive and single-copy sequences made available by the human genome project. This gives us reason to believe that there are yet-to-be-discovered applications of strand-specific hybridization, as well as significant improvements to be advanced for those applications mentioned above. We hope that this short review may serve to encourage imaginative investigators toward that "end".

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