The Next Generation of Metaphase FISH Techniques

The Why and How of directional Genomic Hybridization (dGH)™

Introduction

This application note explains the important differences between dGH and other molecular techniques for measuring genomic structural variation. Key steps in the workflow for performing dGH are outlined, providing insight into the "why" and "how" of this powerful technique.

The "Why" of directional Genomic Hybridization (dGH)™

directional Genomic Hybridization (dGH) is an innovative cytogenetic method enabling the direct visualization of chromosomal changes such as inversions, translocations, deletions, and aneuploidies. dGH is a single-cell method, similar in concept to **fluorescence in situ hybridization (FISH)**, but different in that single-stranded probes target only the parental strand in a single chromatid of a chromosome. In contrast, standard FISH probes bind to both the parental and daughter strands. Small rearrangements, like an inversion, often go undetected with FISH, as there will be no discernible change in the FISH signal pattern (Figure 1).

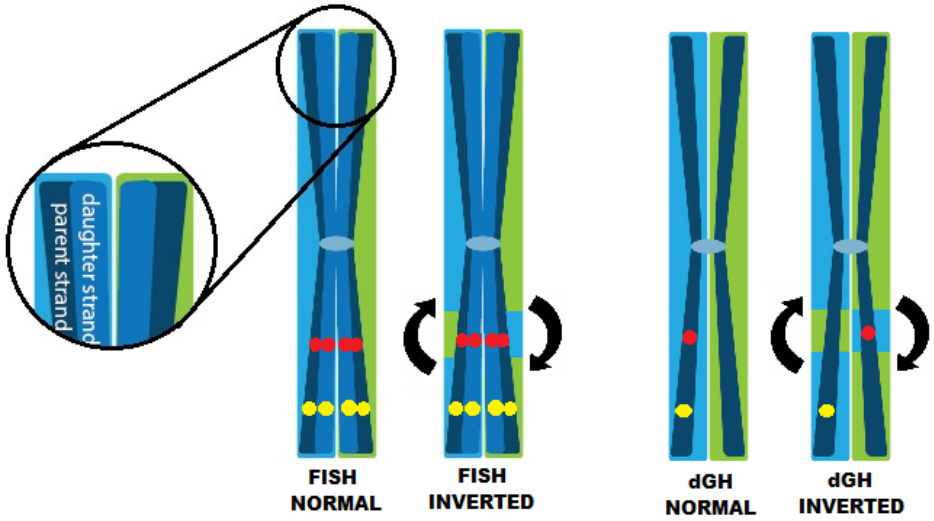

Figure 1: FISH probes hybridize to both parental and daughter strands on both chromatids and may not show any signal pattern change for inversions. Because dGH probes target only one parental strand, they enable visualization of very **small inversions.**

Giemsa banding, or **G-banding**, is a method applied to metaphase chromosomes and represents another option for assaying a cell's genomic status. It does not rely on DNA sequence complementarity but solely on the differential affinity of the stain molecules for regions of DNA rich in either adenine and thymine nucleotides (darkly stained) or cytosine and guanine nucleotides (lightly stained). The varying prevalence of each nucleotide pair, in addition to known heterochromatic and euchromatic regions, produces a recognizable banding pattern for each chromosome.

A drawback to G-banding is the requirement of a fully trained cytogenetics analyst to recognize changes in the genome, and even then, only those that changes that are larger than 5–10 megabases can be resolved. For reference, one of the most high-value oncology gene targets, tp53, spans fewer than 20 kilobases, less than 1% of the assay's resolution limit. Critical changes to individual genes can be too small to be identified by G-banding.

G-banding's status as a legacy method has sometimes made it a default choice as part of regulatory data filings, such as Investigational New Drug Application (IND) submission with the United States Food and Drug Administration (FDA). However, it cannot be relied upon as the sole means of detecting DNA structural changes. It is the applicant who will bear the enormous financial risk of having failed to detect a deleterious abnormality that negatively impacts the viability of a candidate cell therapy.

Next-Generation Sequencing (NGS) techniques are typically population based and can miss rare genetic aberrations occurring at low frequency in a cell population. While single-cell NGS can somewhat negate that issue, it is expensive, time-consuming, and not a direct measurement. One approach meant to mitigate false negatives for these low-frequency variants is to increase coverage depth. However, depending on the assignment software tool used to process the sequencing data, doing this can lead to a counterproductive increase in the false positive rate.

In summary, dGH adds an important level of scrutiny and verification to traditional methods for tracking genome editing outcomes. dGH detects all the abnormalities FISH can, as well as structural changes FISH cannot while greatly exceeding the resolving power of G-banding. That added resolution also extends to its ability to detect numerically small, yet clonal, cell culture populations often missed by NGS.

The "How" of dGH

The following section details the steps used when performing dGH assays, from cell sample preparation through imaging. (Figure 2)

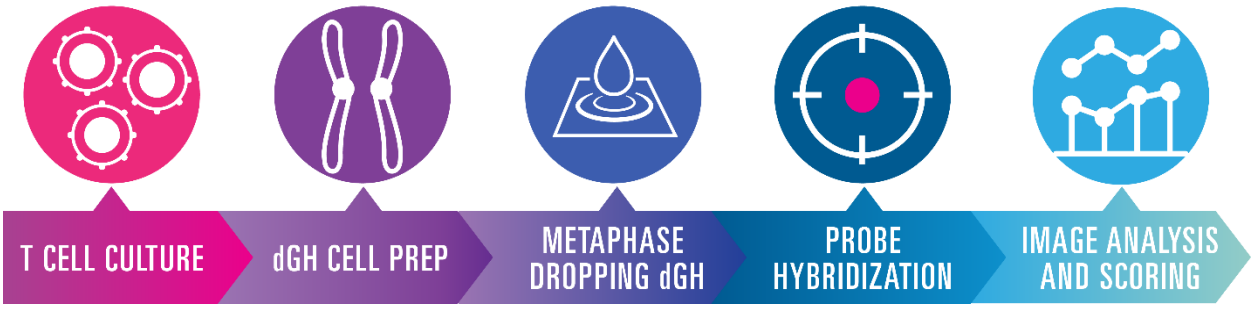

Figure 2: An overview of the entire dGH process from cell culture through image analysis and scoring.

Steps in the dGH Assay

1. Analog Nucleotide Incorporation and Harvest: Nucleotide analogs (dGH™ Cell Prep Additive, Cat. No. DGH-0004) are added to the cell culture medium and are incorporated into newly synthesized DNA daughter-strands as cells proceed through the cell cycle. Colcemid™ (Cat. No. COL-001) is added to block mitosis and cells are harvested at a specific timepoint before the next round of DNA synthesis begins. (Figure 3)

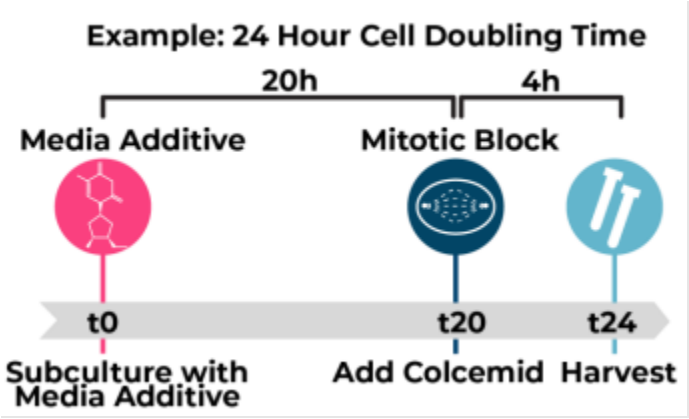

Figure 3: Example cell culture and harvest timeline for preparation of cell sample with a 24-hour doubling time.

2. Metaphase Slide Preparation: Harvested cells are dropped onto glass microscope slides under specific ambient conditions to optimize chromosome spreading. (Figure 4)

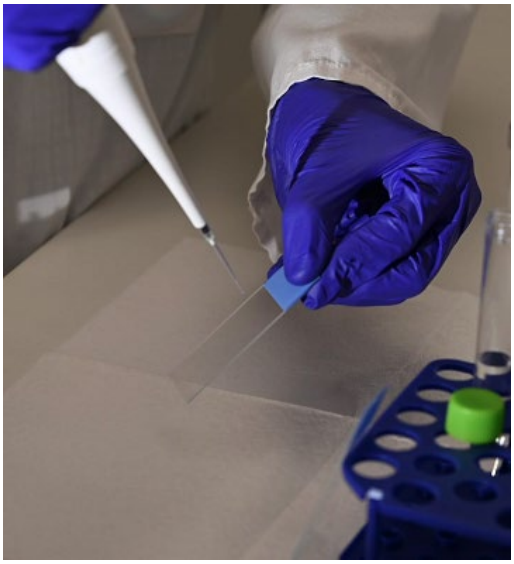

Figure 4: Manually dropping suspended cells onto a microscope slide.

3. Daughter-Strand Degradation: The metaphase slides are incubated with Hoechst 33258. When irradiated with low levels of ultraviolet (UV) light, the daughter strand is preferentially nicked (damaged) at each site of dye-labelled analog. This damage is then subsequently targeted by an exonuclease which leads to degradation of the daughter-strands, leaving behind only the parent strands. (Figure 5)

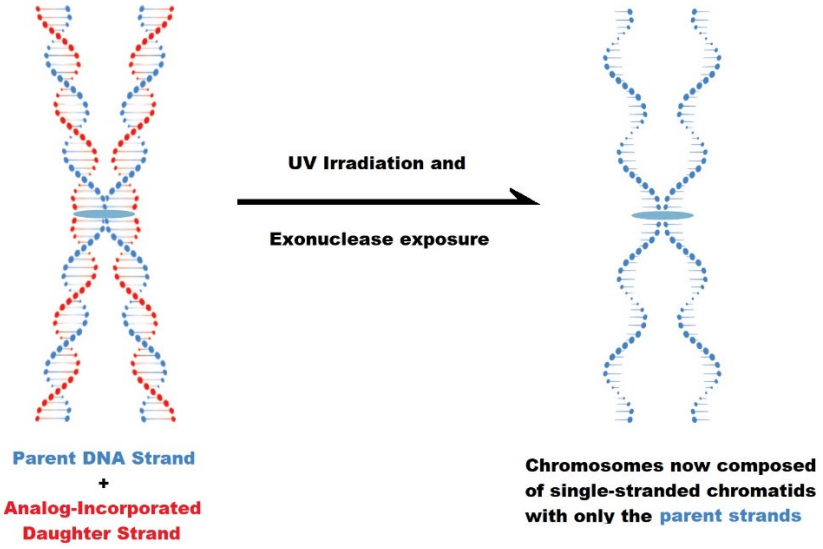

Figure 5: Removal of daughter-strands via UV irradiation and exonuclease exposure

4. Hybridization with dGH Probes: dGH probes are complementary to only one of the two parental strands remaining on each chromosome. If a DNA rearrangement has caused part or all of a probe set's target sequence to move to a new location, this will be revealed by fluorescence in that incorrect locus. (Figure 6)

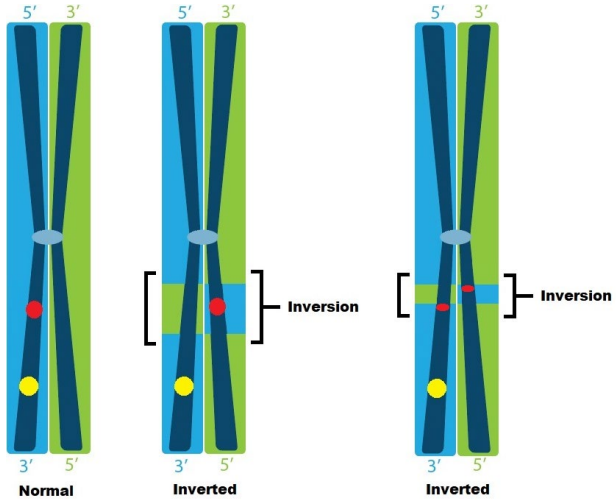

Figure 6: Signal patterns revealed by dGH will vary depending on whether the probe's target locus coincides with a structural rearrangement breakpoint.

5. Imaging and Analysis: The sample is imaged using a fluorescence microscope. In the dGH SCREEN example image below, paint probes show where inversions have occurred. (Figure 7)

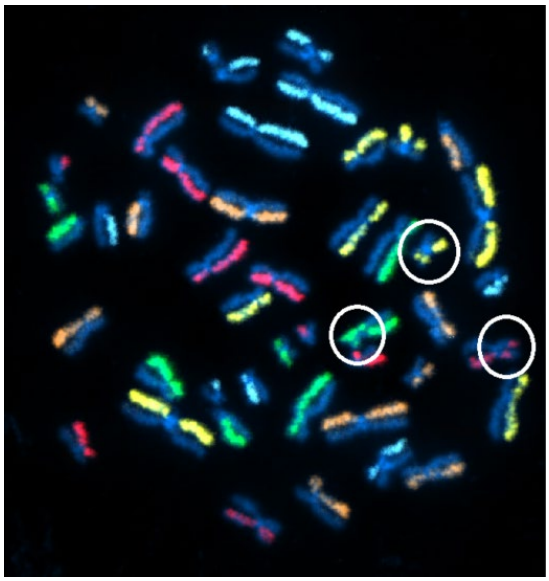

Figure 7: dGH SCREEN detects structural rearrangement sites where fluorescence is observed on both chromatids, as in the circled chromosomes.

Additional Resources

KromaTiD. (2020). What is dGH? [Video]. YouTube. https://youtu.be/T6WQTHBFVEw

dGH™ Cell Prep Protocol. For use in KromaTiD directional Genomic Hybridization Assays

dGH™ in-Site CAR-T Kit 1 Protocol. dGH in-Site Hybridization Protocol v0.9 - EARLY ACCESS

Bothmer, A. et al. "Detection and Modulation of DNA Translocations During Multi-Gene Genome Editing in T Cells" *CRISPR J.* (2020) doi: 10.1089/crispr.2019.0074.

Luxton, J.J. et al. "Telomere Length Dynamics and Chromosomal Instability for Predicting Individual Radiosensitivity and Risk via Machine Learning" *Jrn. Pers. Med.* (2021) doi.org/10.3390/jpm11030188