Single-Cell Measurement of Genomic Inserts and Transgenes

Unbiased, whole-genome tools to assess risks and accelerate gene therapies to market

KromaTiD

Direct, Definitive Genomics

Who We Are

Team of 20 scientists and engineers passionate about directly imaging DNA structure at the lowest possible limit of detection on a single cell basis. Our Focus:

Innovation

- Quantifying location, orientation, size of variants by direct imaging
- Pushing lower the limits of detection with increasing signal strength
- AI automation of imaging and scoring to provide at attractive cost and turn-around time

Execution

- Creating custom assays & probe designs to answer R&D questions
- Supporting GLP Tox Studies/IND filings via structural analysis
- Assuring cell line genomic stability and clonal selection
- Executing assays for clients in rapid fashion

Cytogenetics Support

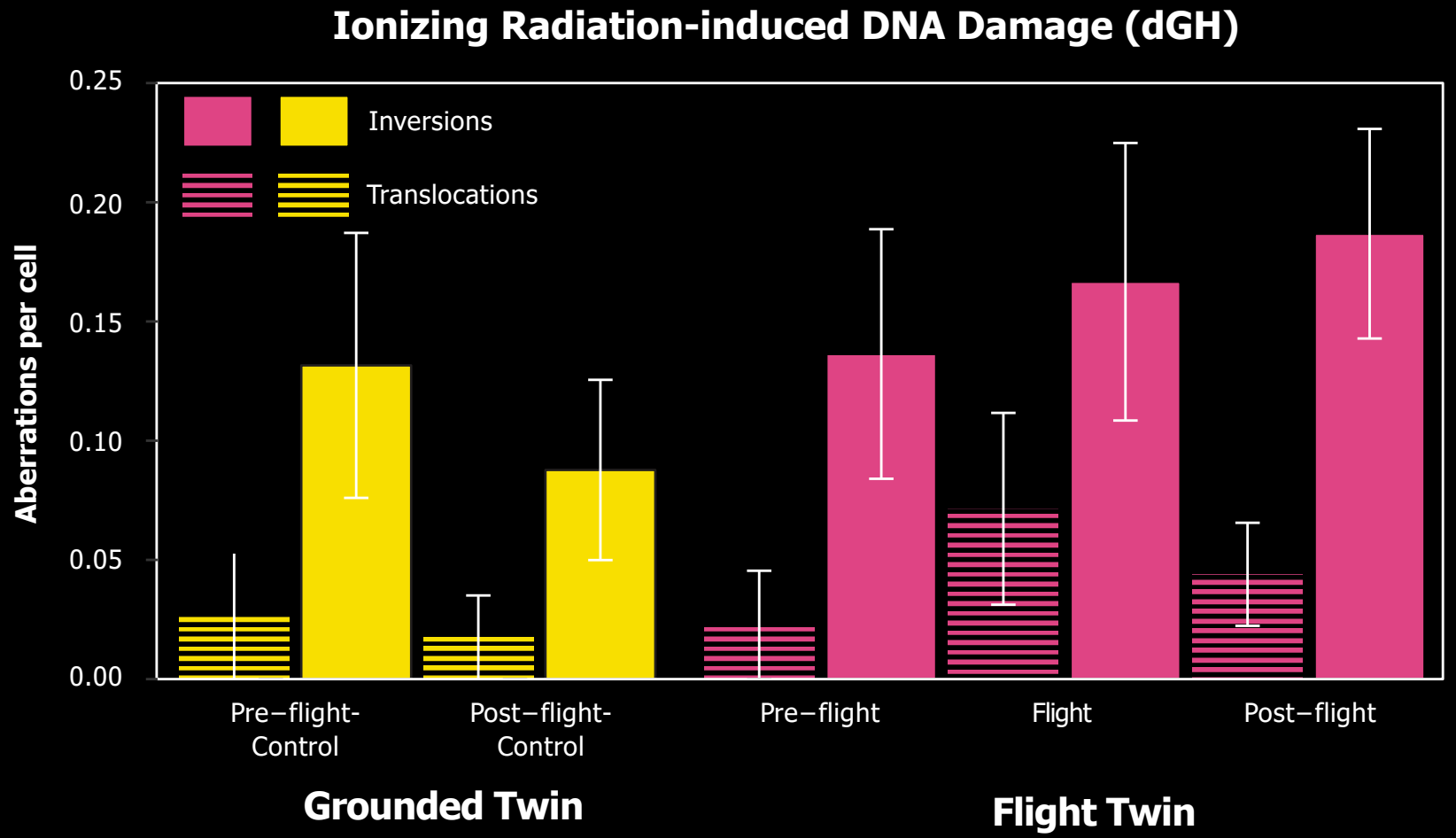
- Provide routine cytogenetics & related testing

Comprehensive, High-Definition Genomic Structural Measurements

dGH is a Fundamental Measurement of Structure

Increased rearrangements during spaceflight consistent with reported radiation doses

Inversions remain elevated, suggestive of on-going instability damage to stem cells, clonal hematopoiesis.



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Genome Engineering and Beyond

Current Capabilities and Applications

Targeted and unbiased assays

- Research support
- QC applications

Pre-IND and IND filing-ready reporting

- Preclinical/GLP Tox study support
- Quantitative
- Orthogonal and complementary to sequencing and other cytogenetic techniques

Oncology
Genome Engineering

Platform Development

Future Potential

Standardized, high-throughput assays

- Automation and AI-driven
- New ways of thinking about variation
- Enabling structural variation metrics

Clinical testing

- Patient qualification
- Clinical subject tracking
- Genomic stability monitoring for gene therapies

Multiple Scalable Diagnostics
Safer Medicines

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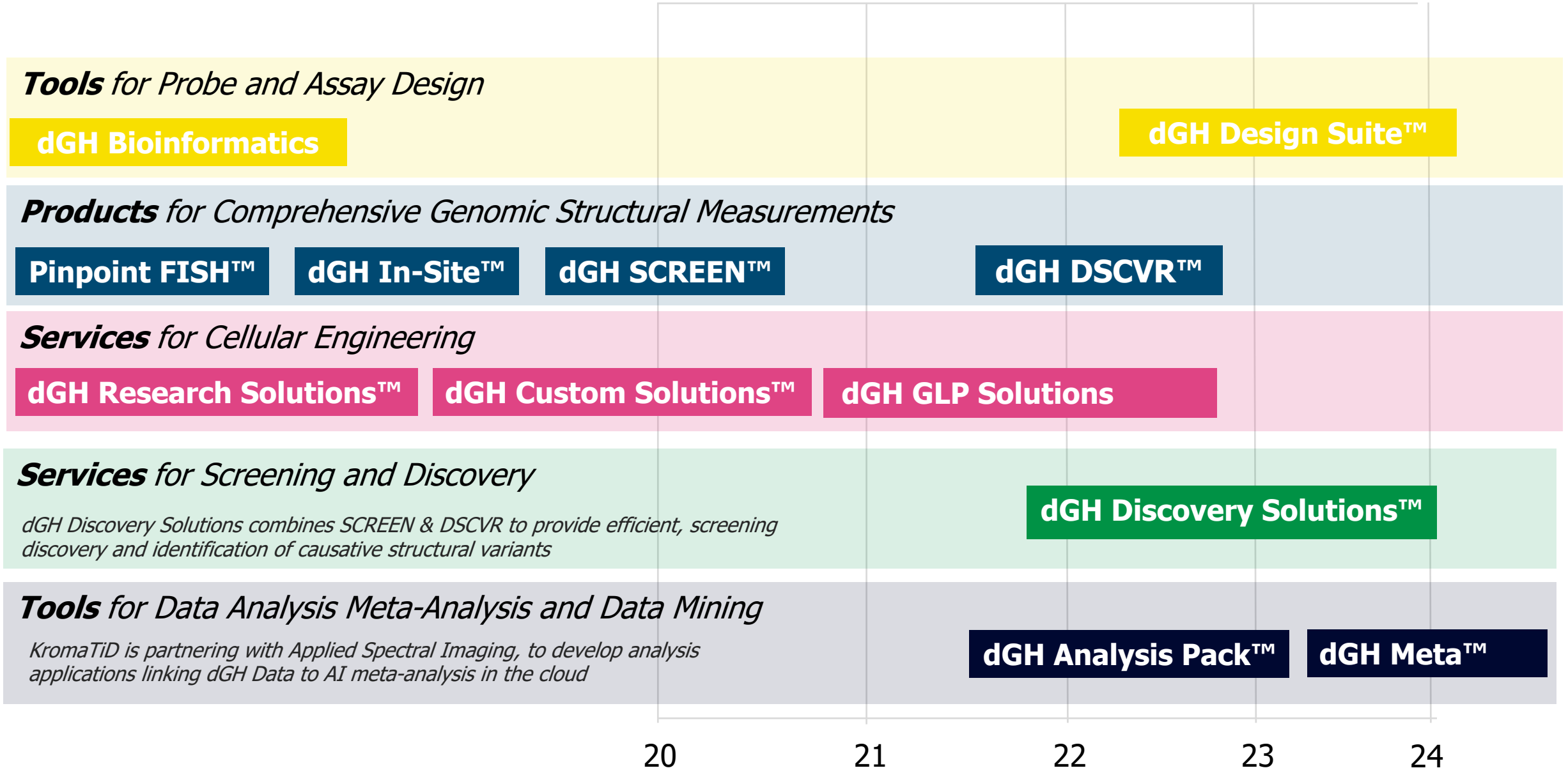
Deep Pipeline of Products, Services and Applications

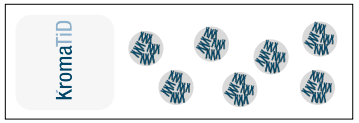


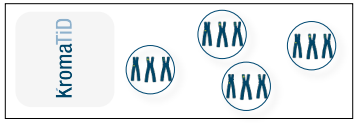
Safe and Effective Gene Therapies Require Further Analytical Developments

"Currently quantitative PCR, e.g. TaqMan qPCR, SYBRGreen qPCR, digital PCR and high-throughput sequencing are the common methods used in integration analysis. **All current methods have significant limitations** in terms of sensitivity, accurate quantification and data interpretation. It has been previously shown that using the currently available methods, the integration vector copy numbers (VCN) were often underestimated. This is because the choice and design of amplification target sequences and the conditions of reaction impact on the specificity and sensitivity of PCR based methods, which makes it difficult to compare data across clinical trials, assays and laboratories."

Zhao, et al. World Health Organization, 2019, *Report on a Collaborative Study for the Proposed WHO 1st International Reference Panel for the Quantitation of Lentiviral Vector Integration Copy Numbers*.

Viral Integration Requires Cellular and Structural Context



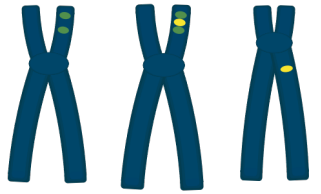


directional Genomic Hybridization

Samples are prepped preserving the genomic structure and cellular context

Chromosomes are gently rendered Single Stranded

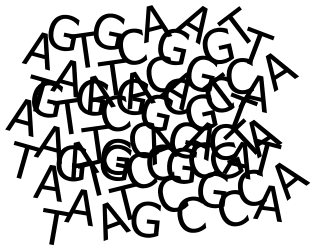
The genomic structure of many cells is mapped through hybridization and analyzed individually



dGH In-Site™

Direct visualization and analysis of integration events

Double Stranded DNA

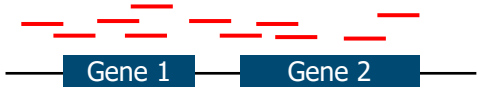


PCR/Sequence Based Methods

DNA from many cells is digested

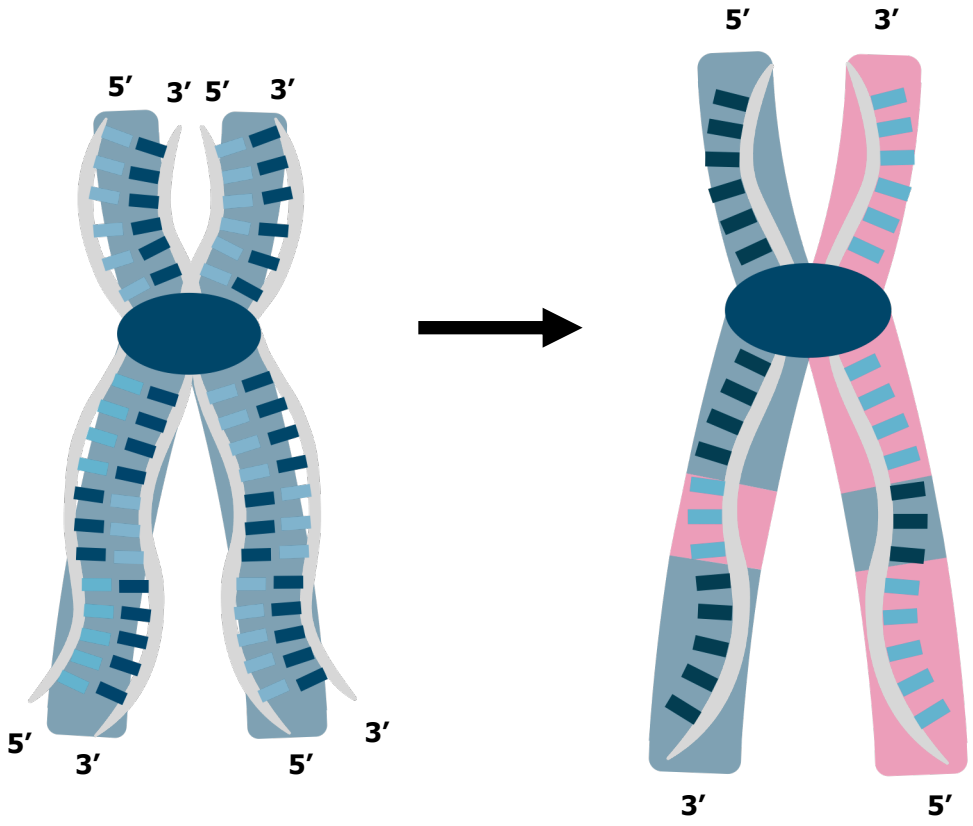
Mixed DNA Fragments from many genomes loses structure and context

Primers targeting areas of interest are amplified



Inferred or calculated structure from pooled DNA

Direct and Robust Visualization of the Genome



Analyte

dGH chromosomes contain **2 strands** of oppositely oriented, **Parental DNA only**—NO Daughter Strands

Single-stranded probes are designed to **target *only* the Watson strand** and *only* unique sequences

1. *Grow cells through one cell cycle*
2. *Incorporate analog during replication*
3. *Strip daughter strands*
4. *Hybridize with proprietary single stranded probes*
5. *Image and analyze*

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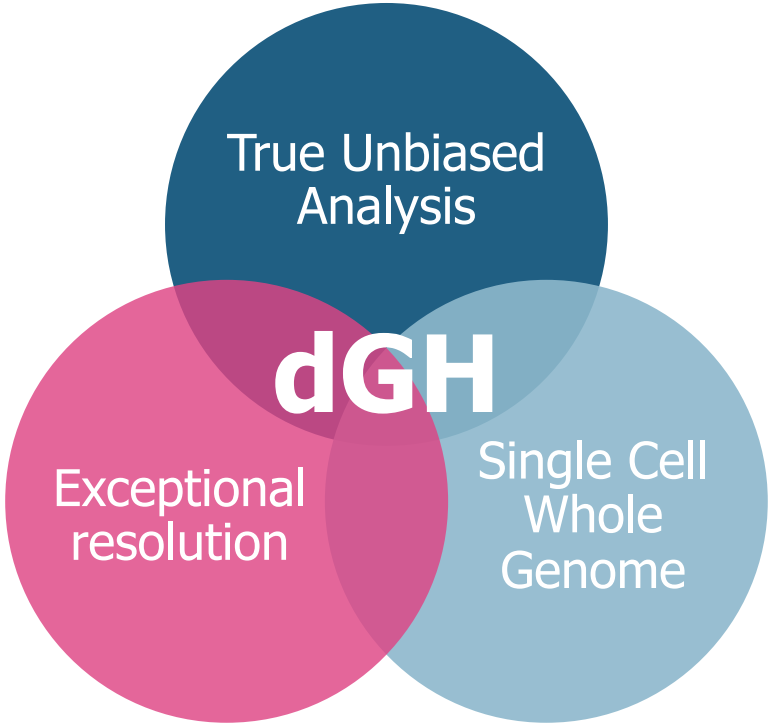
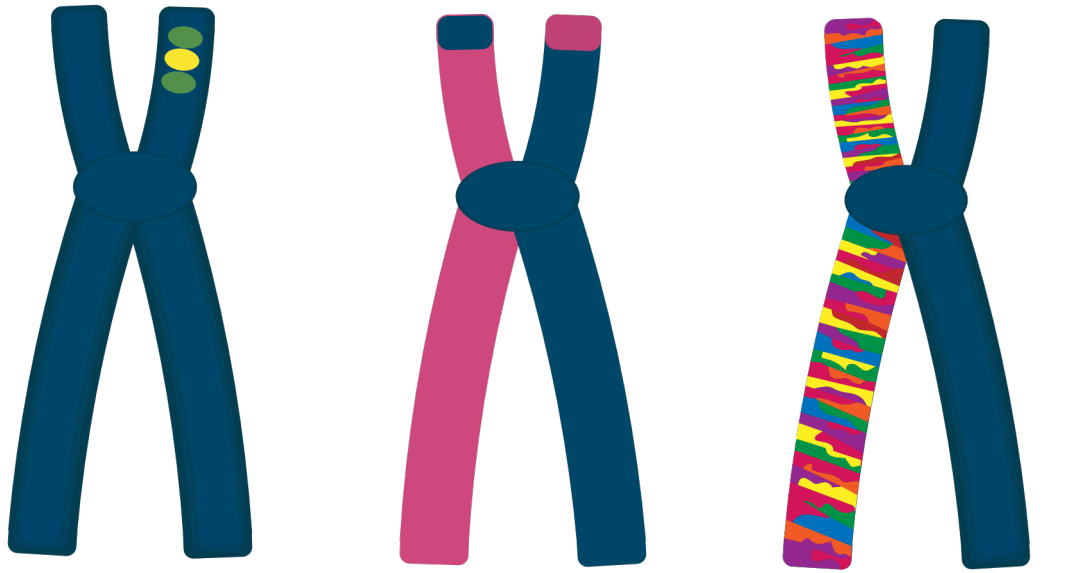
Williams, E., & Bailey, S. (2009). Chromosome Orientation Fluorescence In Situ Hybridization (CO-FISH)

Directional Genomic Hybridization

An unbiased, whole genome, single cell toolset. Map genomes, identify structural variation, and profile structural heterogeneity



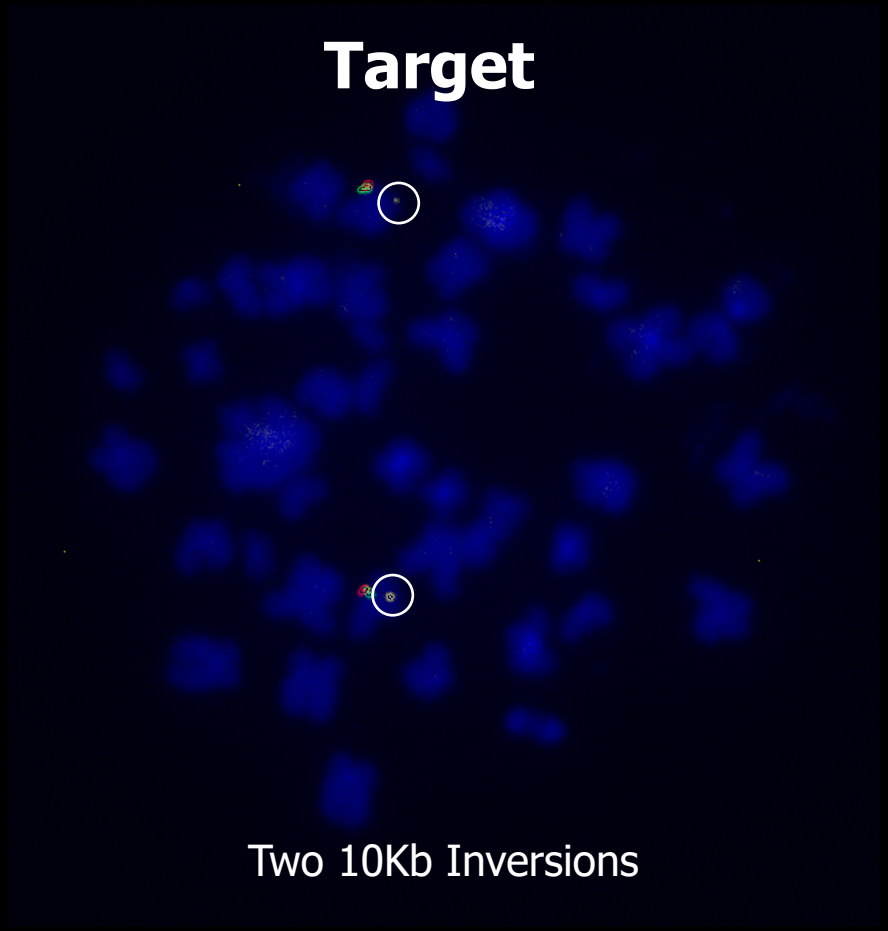


dGH In-Site **dGH SCREEN** **dGH DSCVR**

Development of dGH Screen and DSCVR funded is funded by the NHGRI for discovering and identifying the structural variation that drives and/or influences rare diseases and cancers

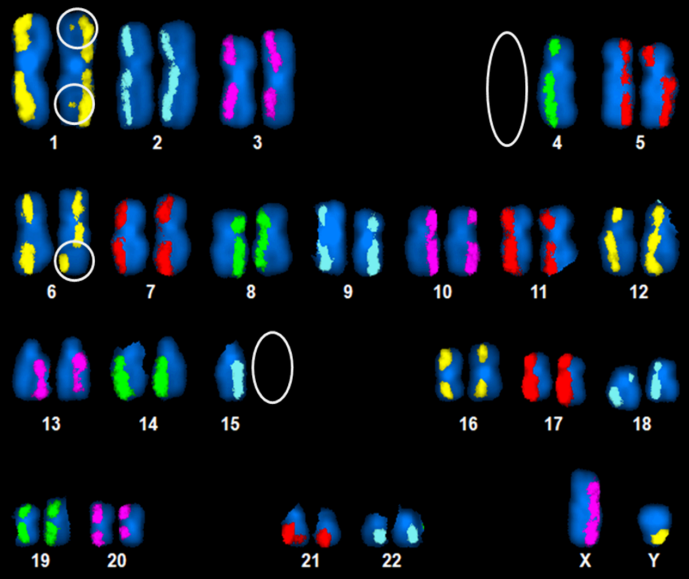
Visualizing Genomic Structure with dGH™

Target



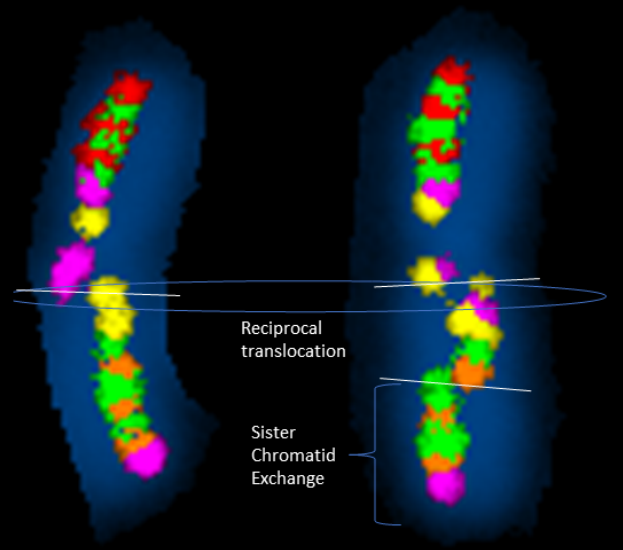
Two 10Kb Inversions

Discovery



Three Inversions and Two Missing Chromosomes

Identification



<1MB Breakpoint Localization

dGH In-Site™ (Targeted)

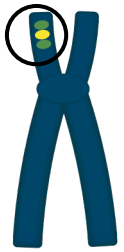
dGH SCREEN™ (Unbiased)

dGH DSCVR™ (Unbiased)

Measuring Integration Events

dGH In-Site™ *quantitative measurement of insertion success and quality*

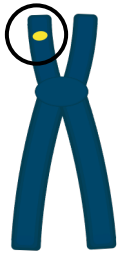
On-Target



Target Chromosome

- Green Probes flank target site
- Yellow signal indicates an on-target insertion
- "Dark" Chromatid Signal indicates inversions

Off-Target



Off-Target Chromosomes

- Yellow Signal without Green Partners indicate Off-Target Insertion
- Off-target green signal indicates translocations

dGH SCREEN™ *Measures genomic instability resulting from potential insertional mutagenesis*

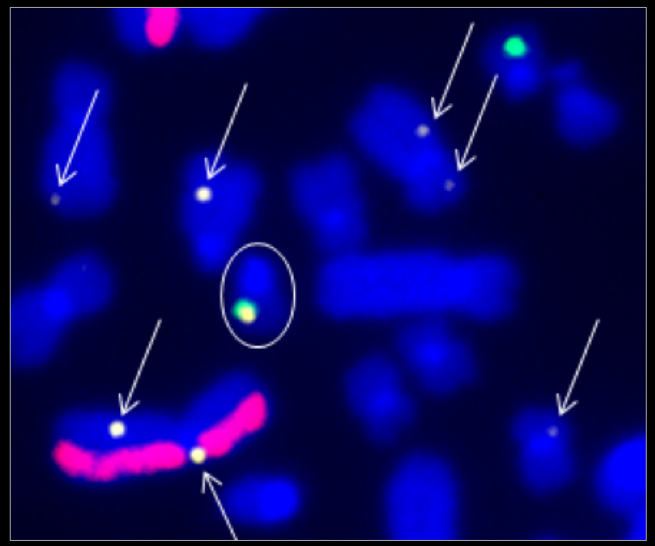
Ch 1, 2, 3

Off-Target Chromosomes

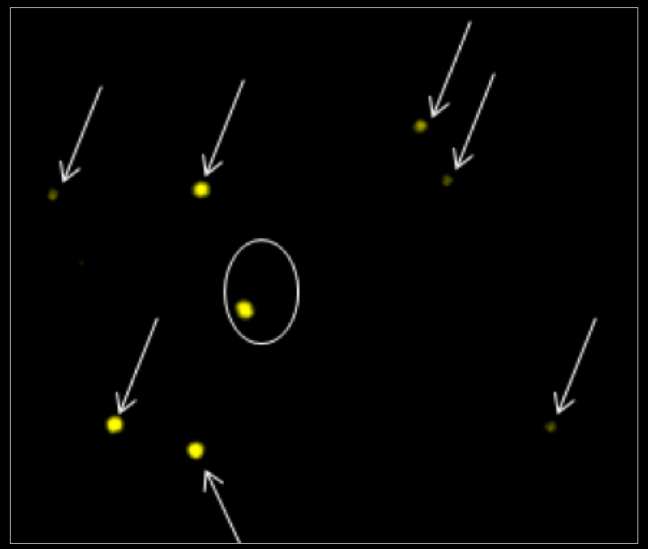
- Paints on any chromosome of interest

1. Prevalence of integration events on-and-off target
2. Location of off target events by chromosome
3. Distribution of integration events by cell

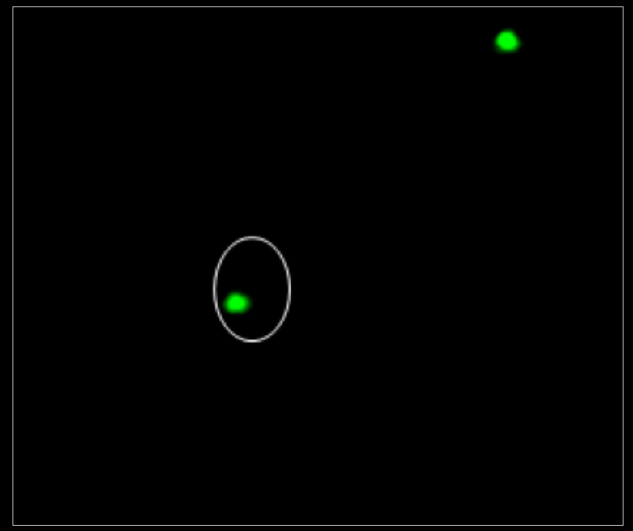
10 Kb Inserts in iPSC: Same Region, 3 Different Color Channels



Fluorescence channels over laid, insert and bracketing probes both visible on one copy of target chromosome and off target inserts visible in multiple chromosomes



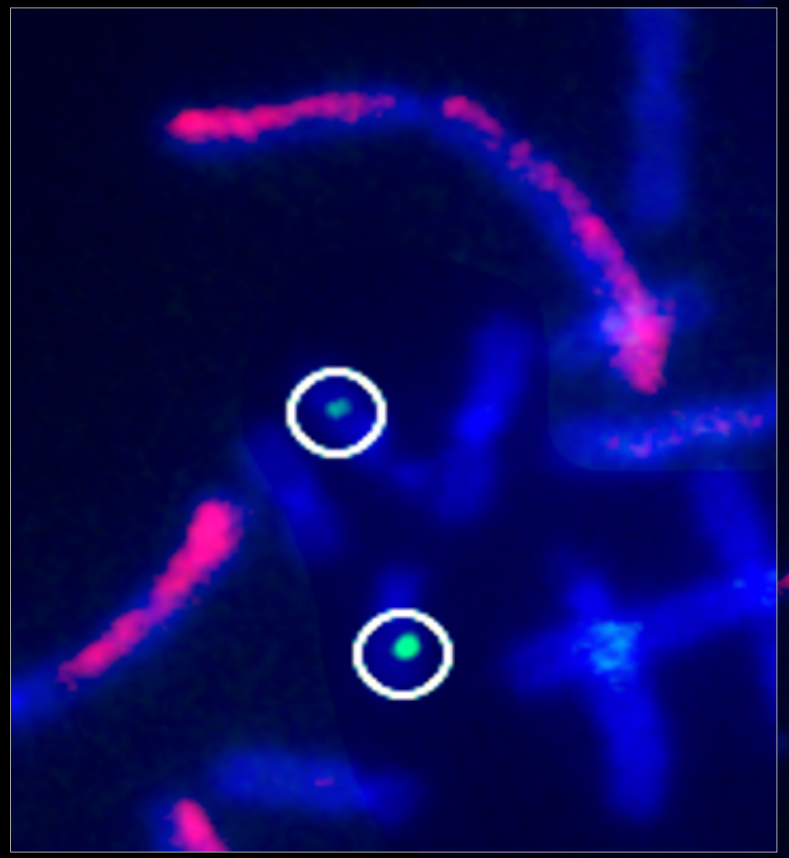
Yellow fluorescence channel, on-target insertion visible on one homolog (circled) and multiple off-target sites throughout genome



Green fluorescence channel, bracketing probes visible on both homologs of target chromosome. Circled green probe signal shows insertion (as seen from yellow channel) while uncircled does not

Same cell with images broken out by fluorescence channel to make the presence of insert more visible. Arrows indicate off-target insertion events.

Control: Donor Human Dermal Fibroblasts



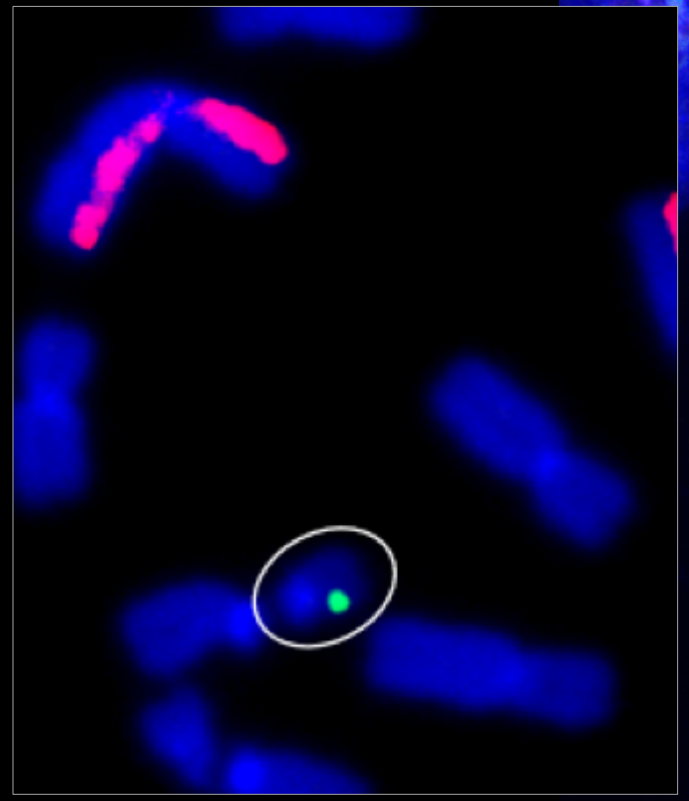
Pink: Chr1,2,3 Dosimetry
Green: Chr 22 On-target bracketing probe

Single Cell Results:

- No inserts detected
- No structural variation at target site
- No Off-Target structural variation

Example of unedited human fibroblast control line cell showing bracketing probes on chromosome 22, dosimetry paints on chromosomes 1, 2 and 3 and no inserts

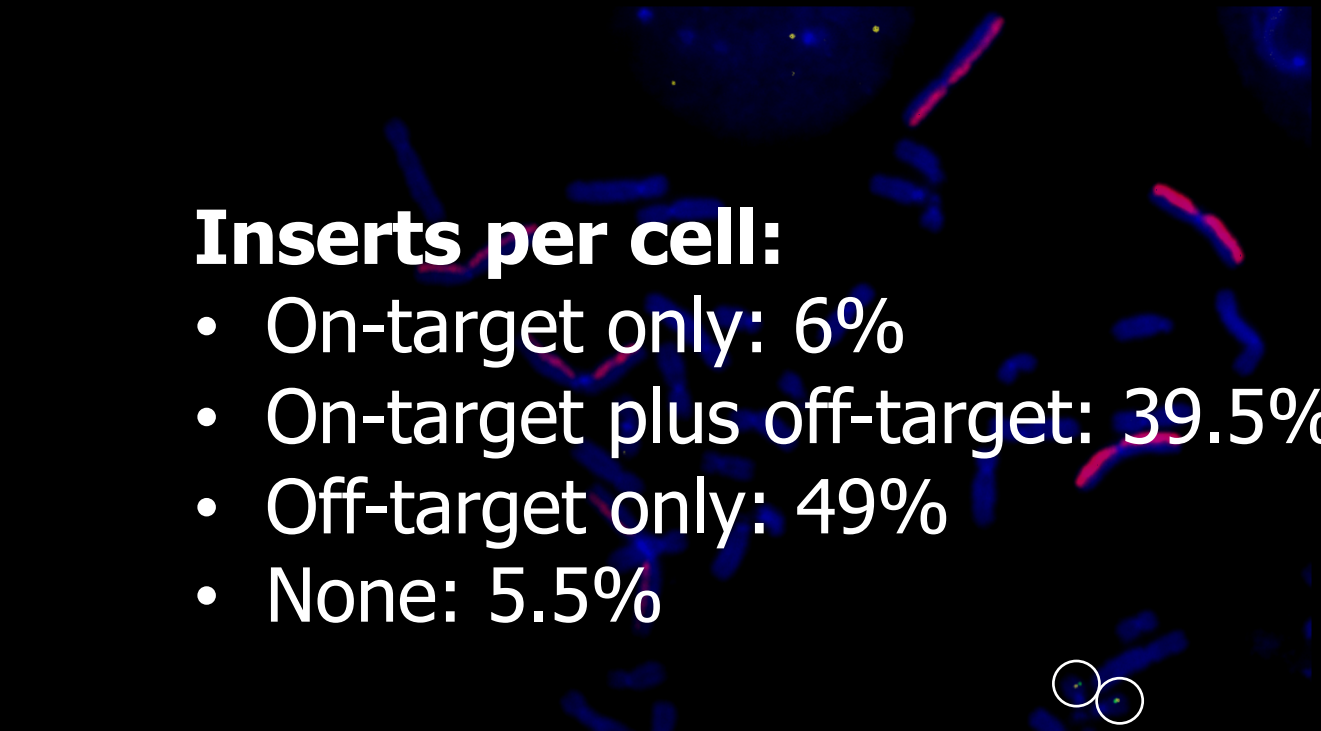
Control: Unedited Derived iPSC



Pink: Chr1,2,3 dGH paint
Green: On-target bracketing probe

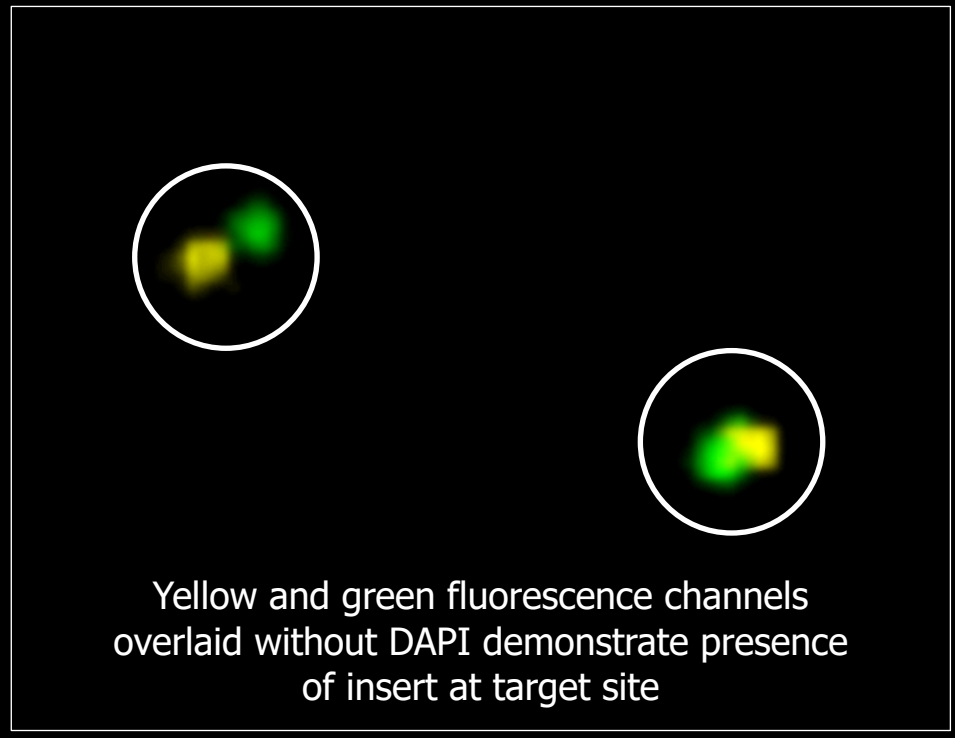
Example of unedited cell with no insert present

Characterization of Integration Events



Inserts per cell:

- On-target only: 6%
- On-target plus off-target: 39.5%
- Off-target only: 49%
- None: 5.5%



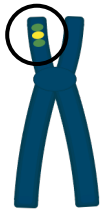
Yellow and green fluorescence channels overlaid without DAPI demonstrate presence of insert at target site

8.3% of on-target inserts were inverted and 1 translocation of the target site was observed

Measuring Integration Copy Number

dGH In-Site™ *quantitative measurement of insertion success and quality*

On-Target



Target Chromosome

- Green Probes flank target site
- Yellow signal indicates an on-target insertion
- "Dark" Chromatid Signal indicates inversions

Off-Target

Off-Target Chromosomes

- Yellow Signal without Green Partners indicate Off-Target Insertion
- Off-target green signal indicates translocations

Internal Size Control Probe

- Yellow signal near Chr8 centromere
- Known size that remains constant

Per cell analysis:

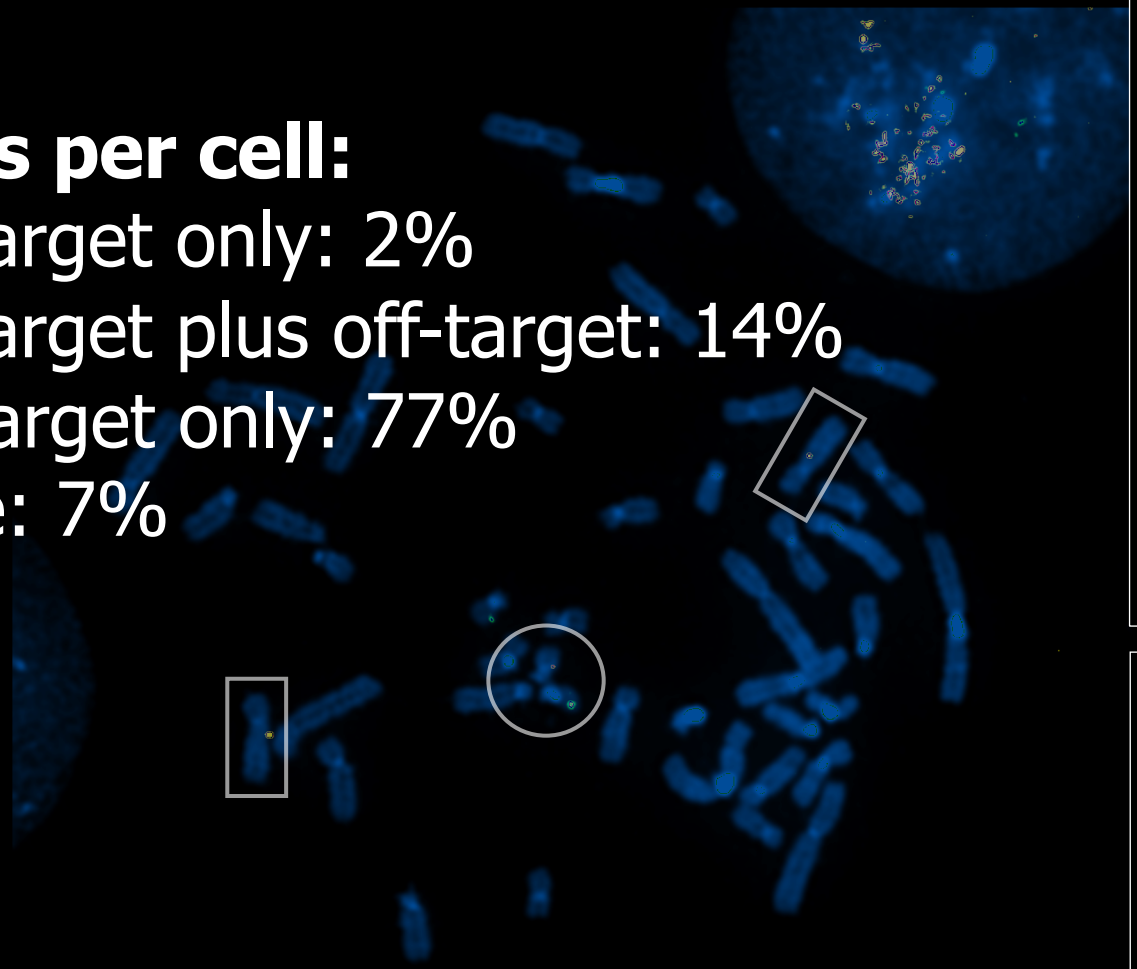
- Normalize all insert and control signals to number of fluorophores per kb
- Compare size and intensity of insert signals to control probe of known size/location
- Calculate estimated integration copy number for each integration event

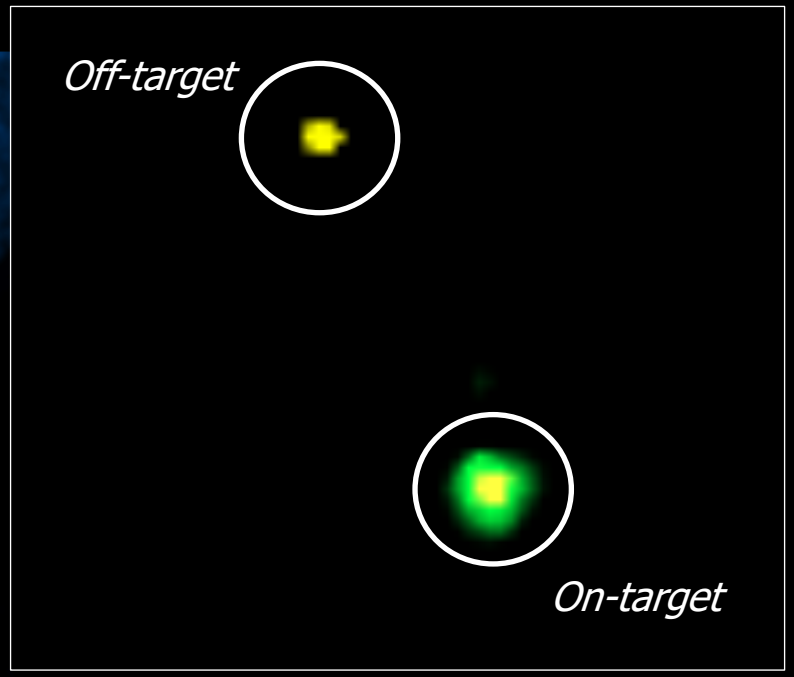
1. Prevalence of integration events on-and-off target
2. Location of off target events by chromosome
3. Distribution of integration events by cell
4. Estimated copy number per integration event per cell

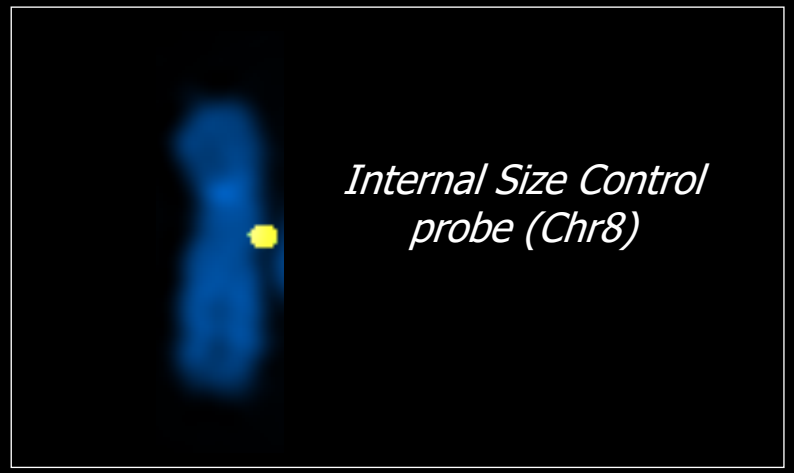
Characterization of Integration Events

Inserts per cell:

- On-target only: 2%
- On-target plus off-target: 14%
- Off-target only: 77%
- None: 7%







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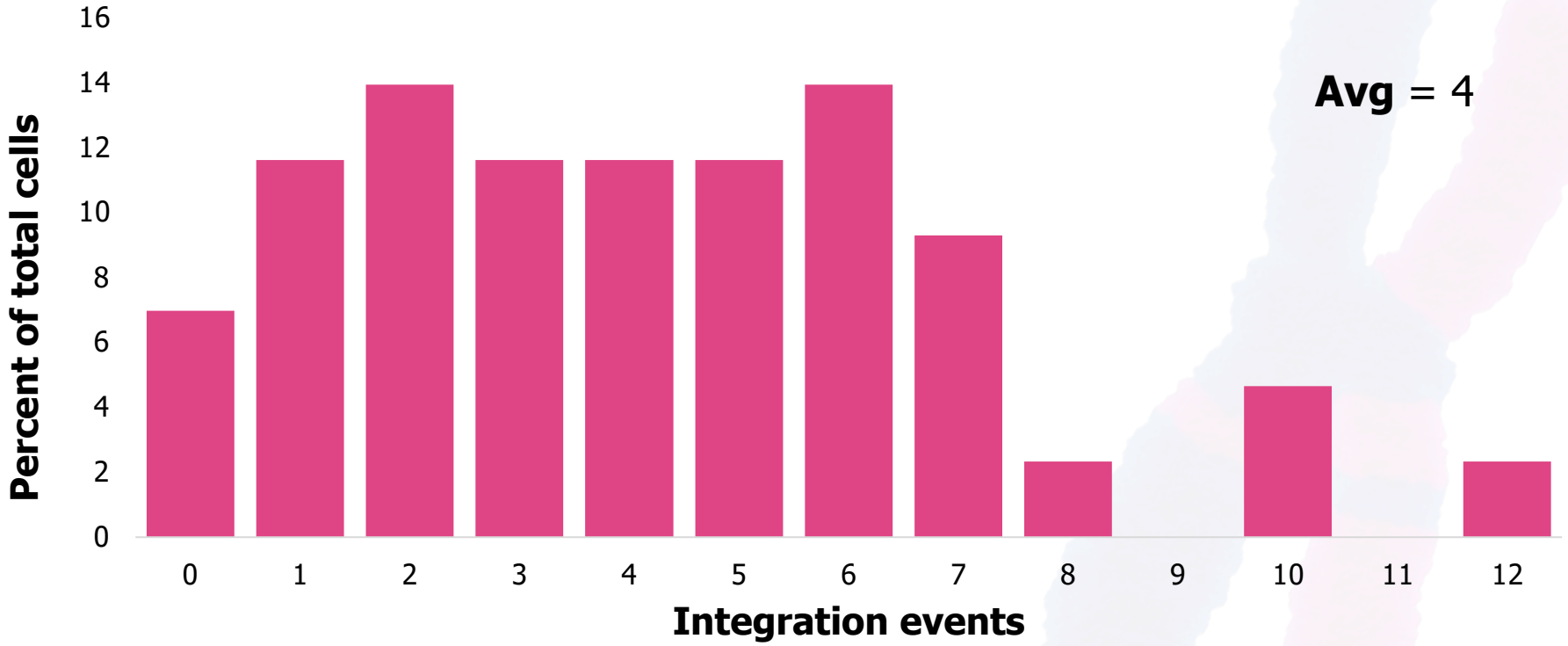
Number of integration events per cell



Represents the total number of integration events per cell. Cells shown as a percent of total cells analyzed.

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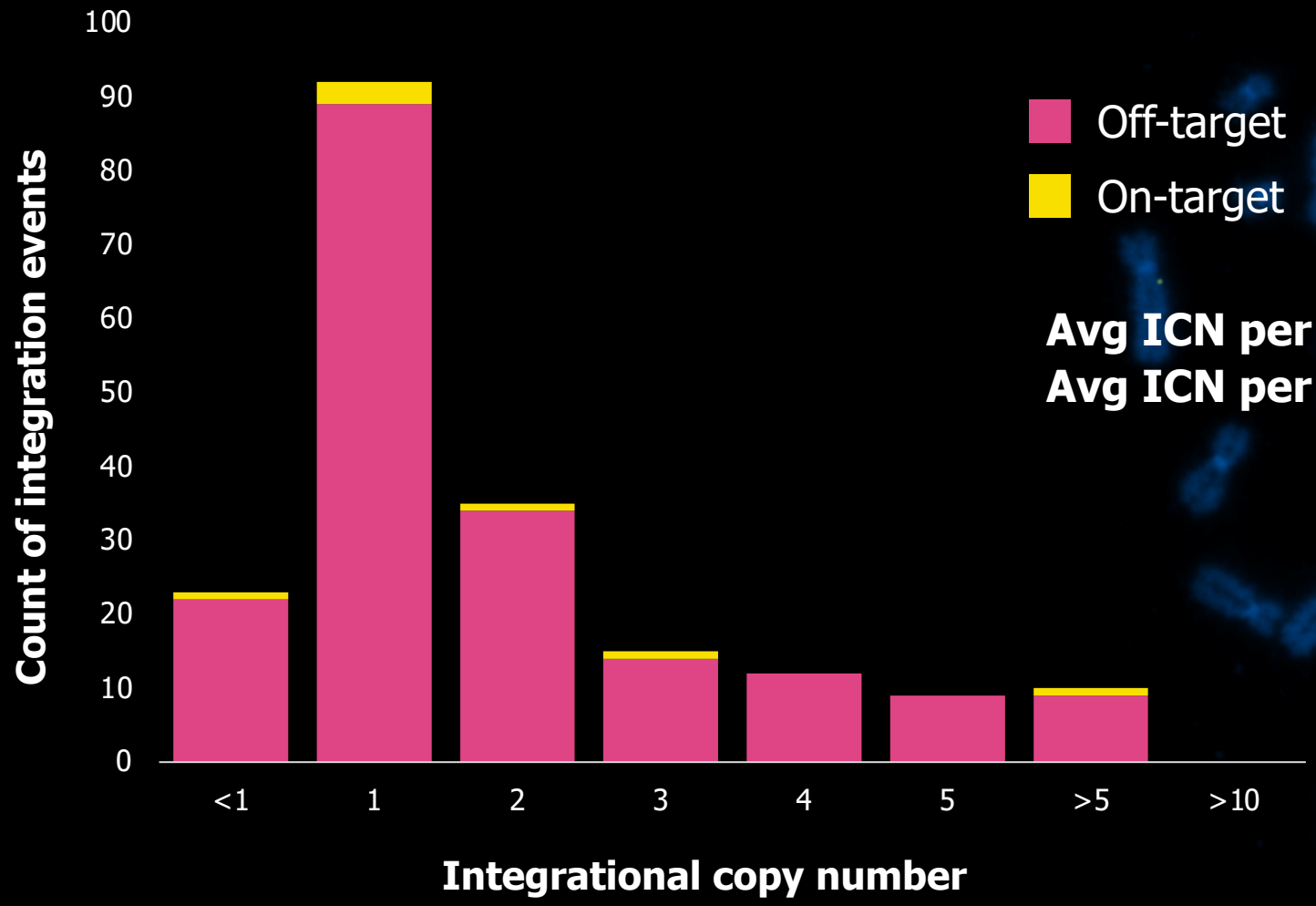
Insertional copy number estimate per integration event



Avg ICN per integration event = 1.6
Avg ICN per cell = 7.8

Measurement Conclusions

Method	Analyte	Qualitative Data Output	Throughput	Quantitative Data Output
dGH in-Site	Many Single-Cells	• On/off target • IE distribution • Whole-genome stability assessment	Low	• Integration events (IE)/cell • Vector copy number (VCN)/cell
TaqMan qPCR	Extracted/Pooled DNA	• Average vector copy number per cell	High	• Integration sites per sample • Estimated VCN/cell • IE location
SYBRGreen qPCR	Extracted/Pooled DNA	• Average vector copy number per cell	High	• Integration sites per sample • Estimated VCN/cell • IE location
Digital PCR (ddPCR)	Extracted/Pooled DNA	• Average vector copy number per cell	High	• Integration sites per sample • Estimated VCN/cell • IE location
Sequencing-Based	Extracted/Pooled DNA	• On/off target • IE gene location	High	• Integration sites per sample • Estimated VCN/cell • IE location

Zhao, et al. World Health Organization, 2019, *Report on a Collaborative Study for the Proposed WHO 1st International Reference Panel for the Quantitation of Lentiviral Vector Integration Copy Numbers*.

dGH Measures

- Rate of **on vs. off target** insertions in a population
- Number of **integration events** per cell
- Estimated **copy number** per integration event
- **Inversions** of inserts or other target genes
- Background levels of **rearrangements** due to editing

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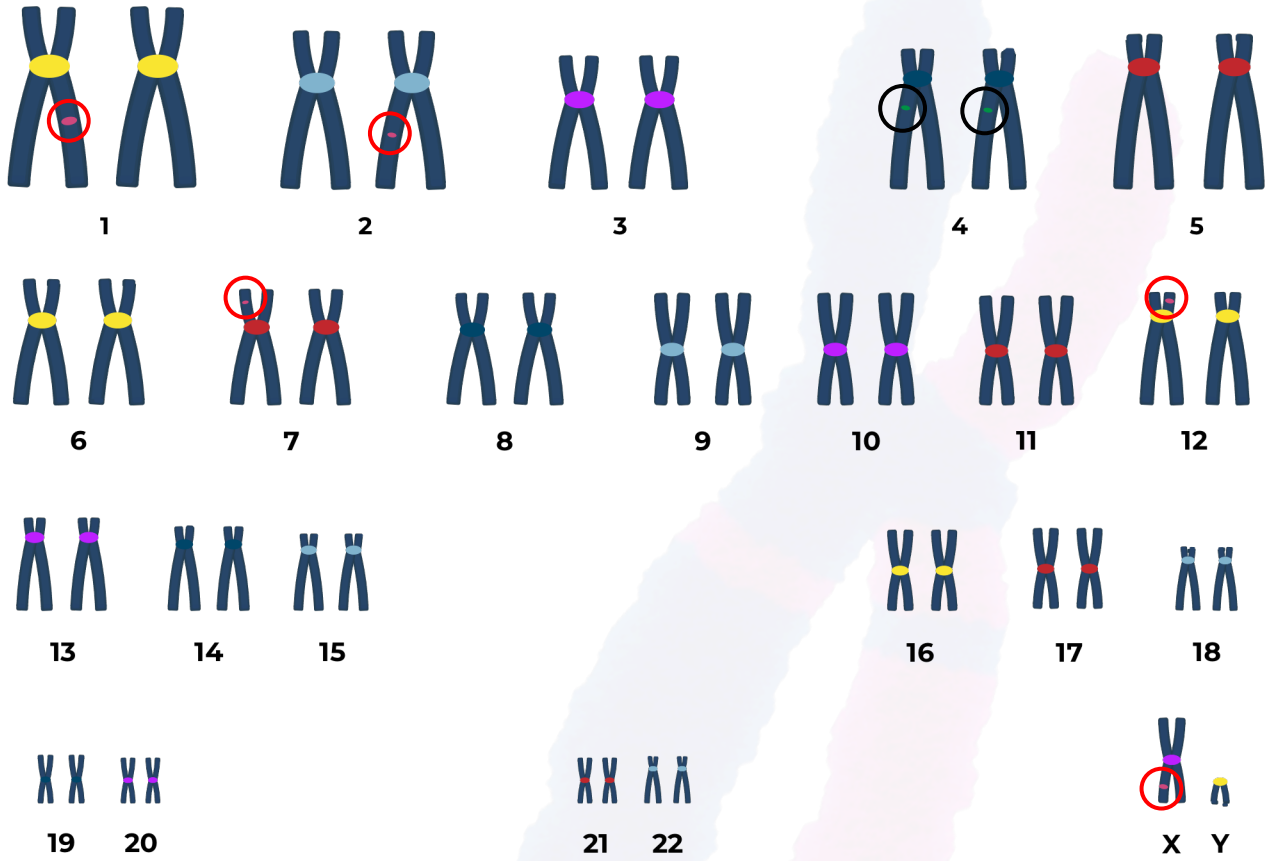
Where We're Going

- Label chromosome with unique probes at the centromere, group by size and color
- Internal sizing control probe in different color
- Genome copy number per cell determined by RFU
- Machine learning expedites scoring and increases throughput



Example of dGH in-Site 2.0 assay organized karyographically. Chromosome color groupings and morphology enable identification, which allows for insert localization to the p or q arm of any chromosome. In this example, insert is labeled pink, and all sites of integration are circled in red. The albumin gene on ch 4, which is used as an internal control, is circled in black.

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Working with KromaTiD is Easy

Client	KromaTiD
1. Provide target(s) and measurement objectives	--
--	2. Propose probe and assay design & quote
3. Approve design & quote	--
--	4. Set up project team, make probes & schedule
5. Send samples	
--	6. Receive and prep samples
--	7. Prelim imaging & scoring rules (~10% of cells)
8. Approve scoring rules	--
--	9. Complete imaging & analysis
--	10. Upload data and report
11. Review data and report	11. Review data and report

Project Management

- Focused project management resources on client projects
- Regular, systematic updates and reporting
- Right-first-time and on-time commitment
  - First analysis: 10w
  - Repeat analyses: 4w or less

Thank You..

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