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CRITICAL REVIEW

A framework for the clinical implementation of optical genome mapping in hematologic malignancies

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Abstract

Optical Genome Mapping (OGM) is rapidly emerging as an exciting cytogenomic technology both for research and clinical purposes. In the last 2 years alone, multiple studies have demonstrated that OGM not only matches the diagnostic scope of conventional standard of care cytogenomic clinical testing but it also adds significant new information in certain cases. Since OGM consolidates the diagnostic benefits of multiple costly and laborious tests (e.g., karyotyping, fluorescence *in situ* hybridization, and chromosomal microarrays) in a single cost-effective assay, many clinical laboratories have started to consider utilizing OGM. In 2021, an international working group of early adopters of

OGM who are experienced with routine clinical cytogenomic testing in patients with hematological neoplasms formed a consortium (International Consortium for OGM in Hematologic Malignancies, henceforth "the Consortium") to create a consensus framework for implementation of OGM in a clinical setting. The focus of the Consortium is to provide guidance for laboratories implementing OGM in three specific areas: validation, quality control and analysis and interpretation of variants. Since OGM is a complex technology with many variables, we felt that by consolidating our collective experience, we could provide a practical and useful tool for uniform implementation of OGM in hematologic malignancies with the ultimate goal of achieving globally accepted standards.

1 | INTRODUCTION

Until recently, the clinical evaluation of genome-wide structural variation (SV) has been performed primarily by karyotyping (herein chromosome banding analysis, CBA). For the purpose of this manuscript, we will use the term SV as an inclusive term, including both classic SV (larger than 500 bp) and numerical chromosomal changes as done by others.[1] The detection of structural changes associated with cancer was first reported in the late 1960s and 1970s and began to enter widespread use in the 1980s.[2,3] Hallmark chromosomal abnormalities identified by CBA in various hematologic malignancies have been at the foundation of the nosological classification systems and are integral to diagnosis, prognosis, clinical management, and choice of treatment regimens.[4–6]

1.1 | Conventional cytogenetic testing

CBA, as a technique, has many strengths that make it useful for detecting copy number abnormalities (CNAs) and SVs (balanced and unbalanced rearrangements). It is the only method to visualize the genome in its native state, without breaking it apart and then mapping its segments to a reference genome. The visualization of metaphase chromosomes as individual structures ("single cell" analysis) provides a "whole genome" view of the structural composition of the genome that is largely unparalleled, irrespective of sequence composition (e.g., constitutive heterochromatin, centromeres and telomeres). A key benefit of the single cell nature of CBA includes clonal analysis of cancer samples that reveals the evolution and clonal heterogeneity of tumors with relative ease and at low cost (simply by analyzing an adequate number of cells).

However, various limitations of CBA exist. A principal drawback is the requirement for actively dividing (live) cells, which necessitate cell culturing, to be able to perform metaphase analysis. Some hematologic cancer cells do not divide well in culture and, without specific mitogens, cannot be reliably analyzed using CBA. In addition, cell culturing may introduce a bias due to preferential division of cells that have a selective growth advantage. Reference laboratories that receive shipped specimens face additional challenges that affect cell viability such as delayed shipping and/or adverse temperature exposure.

The size of SVs that can be identified by CBA is limited by the technical constraints of the light microscope and the poor chromosomal morphology often obtained from cancer samples. In base pair terms, the lower limit of resolution for karyotype analysis in hematological malignancies is considered to be ≥10 Mb. Genomic aberrations below this threshold are "submicroscopic" and are not expected to be detected by CBA. Clinical cytogenetic laboratories have adopted ancillary molecular/cytogenetic techniques like fluorescence *in situ* hybridization (FISH) and multiplex ligation-dependent amplification (MLPA) to detect submicroscopic abnormalities. However, these techniques utilize a pre-defined set of probes that only interrogate specific targeted regions. Chromosomal microarray analysis (CMA) is also useful for identifying CNAs beyond the resolution of CBA but cannot detect balanced structural changes, such as translocations and inversions nor the location or orientation of inserted genomic material. While FISH, MLPA, and CMA play important roles in cytogenetics laboratories, in general, they are ancillary techniques to the karyotype, especially for many hematologic malignancies.

The variable chromosomal morphology of leukemic cells and the subjective assessment of banding patterns contribute to another important limitation of CBA, namely "rearrangement ambiguity." The analysis of banded chromosomes relies on a "process of elimination" where abnormal chromosomes are compared to their normal counterparts and the rearrangement type is deduced. This works very well for "simple abnormal" and relatively non-complex karyotypes. However, as both the number and complexity of rearrangements and imbalances increases, the ability of CBA to unambiguously identify specific rearrangements and match the corresponding rearrangement partner is considerably reduced. Terms, such as marker chromosomes ("mar") and additional material of unknown origin ("add"), are commonplace in clinical cytogenetic reports of cancer samples and have also led to a battery of ancillary tests (both molecular and cytogenetic) to help better detect clinically relevant aberrations. However, as our understanding of the genetic etiology of hematologic malignancies and treatment options continues to expand, so do the requirements for biomarker testing.

1.2 | Optical Genome Mapping—The technique

Optical Genome Mapping (OGM) is a genome mapping technique that can detect SVs across the entire genome at high resolution. OGM is performed by extracting ultra-high molecular weight (UHMW) DNA (median N50 ≥150 kb), enzymatically labeling the DNA at specific

DNA motifs that repeat approximately every 6 kb, and imaging individual DNA molecules as they run through a nanochannel flow cell. DNA images are converted to digital molecules and then bioinformatically assembled to create a genome analysis with a complete assessment of structural and copy number variation (for review, see Smith et al.[7]). Tissue culture and/or DNA amplification steps are not required during hematologic sample processing thus eliminating the risk of selective advantage culture bias and PCR artifacts often encountered using other techniques.

The patterns generated from the labeled molecules can be thought of as being analogous to "chromosome bands," albeit at a resolution 100×–20 000× greater than attained by CBA (increased resolution level depends on the type of abnormality—see Table 1 in Smith et al. [7]) The net result is a high-resolution genome-wide analysis that, in a single assay, matches or exceeds the diagnostic scope of multiple combined techniques currently used in clinical cytogenetics laboratories.

1.3 | Concordance studies between conventional cytogenetics and OGM

Several recent publications have compared the performance of OGM to standard cytogenetic approaches. These include a general assessment of OGM in various hematologic malignancies[8–11] as well as focused evaluations in specific patient cohorts such as Acute Myeloid Leukemia (AML),[12] Pediatric AML,[13] Myelodysplastic Syndromes,[14] Acute Lymphoblastic Leukemia,[15–17] and Chronic Lymphocytic Leukemia.[18,19] These studies have all demonstrated that OGM has equal or better sensitivity and resolution for the detection of diagnostic and prognostic abnormalities in hematologic malignancies with relatively few discordances. As such, clinical laboratories are now pursuing OGM for detection of sentinel cytogenomic abnormalities in patients with hematological neoplasms. Laboratories considering implementation of OGM into the clinical workspace may consider 1) running OGM as a first-line tool with appropriate reflex testing where necessary or 2) using OGM in combination with CBA to replace ancillary testing such as FISH panels and CMA (which would still be a cost-effective approach). Given that OGM is a very new technique in the clinical arena, there is a distinct lack of guidance regarding clinical validation and implementation. In response to this need, we established an international consortium of early adopters of OGM to formulate an experience-based framework for clinical use of OGM in patients with hematological malignancies.

TABLE 1 Performance calculations for methodological validation.

Parameter	How to calculate
Sensitivity/positive percentage agreement	TP/(TP + FN)
Specificity/negative percentage agreement	TN/(TN + FP)
Positive predictive value	TP/(TP + FP)
Negative predictive value	TN/(TN + FN)
Accuracy	(TP + TN)/(TP + TN + FP + FN)

Abbreviations: FN, false negative (type 2 error); FP, false positive (type 1 error); TN, true negative; TP, true positive.

1.4 | The OGM framework

The Consortium was formed by an international group of clinical cytogeneticists who have experience in CBA, FISH, and CMA, and recently in OGM for the detection of genomic abnormalities in patients with hematologic neoplasms. Looking at the lack of standardization in validation, analysis criteria and reporting practices that plagued clinical testing using CMA when it was introduced, we believe it prudent to take a proactive approach to address similar issues when introducing OGM into the clinic. The primary purpose of formulating this comprehensive framework is to assist laboratories with a more uniform implementation of OGM in hematologic malignancies with the aim of achieving globally accepted standards. While some quality and reporting requirements will differ between jurisdictions, this framework is meant to consolidate practice and provide guidance for laboratories implementing OGM in three specific areas: methodological validation, quality control considerations and analysis and interpretation of variants. OGM is a complex technology with many variables and a consensus framework has been compiled based on the collective experience of the members of this international Consortium. It should be acknowledged that while this document intends to provide guidance, it is not an official guideline. Official guidelines are usually published once a technique is well established and based on the growing complexity of genome analysis in different hematologic malignancies, specific guidelines for groups of hematologic malignancies may be required (e.g., myeloid vs. lymphoid). While we believe that most of the recommendations in this framework will continue to be useful, software updates and disease specific guidance may require future adaptation.

2 | VALIDATION

Many aspects need to be considered and addressed before implementing a new genomic technology in a diagnostic setting. Key aspects include:

1. Assessment of clinical utility: Evidence should exist to show how the technology provides clinical information to patients that meet or exceed current standards. Such evidence typically derives from published studies performed by early adopters but could also be self-initiated.
2. Proof of principle study: The laboratory should assess whether the technology is feasible and functions as anticipated. Protocol optimizations should be performed as necessary.
3. Method validation: It should be verified that the new test meets the performance characteristics predefined with regard to the application.

4. Clinical validation: Should be performed to evaluate diagnostic/clinical performance in unbiased blinded analysis of patient samples.
5. Implementation: The new assay implementation should include additional controls and should follow established regulatory requirements and laboratory policies.

In this section, we give a detailed description of the different steps of this process for laboratories intending to implement OGM into the diagnostic setting for hematological malignancies. It goes without saying that OGM is a complex genomic test and that a good knowledge of cytogenetics and molecular genetics, as well as an understanding of the biology and genetics of hematologic malignancies is essential for the accurate technical evaluation, clinical interpretation, and reporting of OGM variants. The general consensus of the Consortium regarding clinical implementation of OGM for patients with hematological malignancies is that OGM is a robust technique with an end-to-end workflow (including bioinformatic processing) provided by the manufacturer (Bionano Inc). Laboratories with experience in cytogenetic and molecular techniques should find the technical implementation straightforward.

2.1 | Clinical utility

Clinical utility can be defined simply as the value of a novel technology or practice for patient care. For OGM, the evidence for clinical utility can be gathered by the laboratory during method validation (see below) or from published literature. With several recent studies demonstrating the increased resolution and better diagnostic sensitivity in hematologic malignancies of OGM compared to standard of care (SOC) methods, the laboratory may consider these published reports as sufficient evidence to establish clinical utility. In addition, the definition of clinical utility may also be expanded to consider aspects such as logistical or financial considerations. For example, does OGM replace or complement several existing technologies, improve turn-around time, or even ameliorate staffing or laboratory workflow issues. These additional considerations can also be included in an assessment of clinical utility.

2.2 | Proof of principle

Before starting validation of a new technology, the laboratory has to evaluate if the given technique is fit for the intended use which for the purposes of this manuscript is to perform comprehensive cytogenomic analysis in patients with hematological malignancies. Using literature references as a guide, the laboratory can initiate a pilot study or a more expanded research study to accomplish this goal. OGM can certainly be used for other indications (e.g., constitutional and prenatal testing) as well as hereditary cancer predisposition analysis—but those applications are considered outside the scope of this framework. The establishment of standard protocols that generate high quality data and documentation of assay "final conditions" is important to perform at this stage and prior to embarking on assay validation.

2.3 | Method validation

A methodological validation for OGM as a laboratory developed test (i.e., non-FDA cleared test) requires several steps including: (1) determining the type and number of samples to be tested; (2) establishing test performance (e.g., analytic sensitivity, analytic specificity, accuracy and precision); (3) demonstrating test reproducibility; and (4) determining the lower limit of detection (LLOD). This framework is intended to describe the basic outline of the requirements needed to validate OGM for the testing for hematologic malignancies. Furthermore, laboratories may wish to implement OGM as a supplement to conventional methods initially or as a replacement for them depending on the laboratory's needs and the specific hematological malignancies tested.

2.3.1 | Validation cohort size and specimen type

OGM is a novel, genome wide assay that requires a sufficient number of representative samples for methodological validation. Jennings et al.[20] calculated that a sample size of 59 would produce sufficient data to accurately determine assay parameters (e.g., sensitivity, specificity, accuracy, and precision) for complex genomics assays. While this number of samples provides a general methodological validation of the technique, testing additional samples with specific clinical indications is also advised to make sure the laboratory is comfortable with the analysis and reporting peculiarities of each disease (see below section 2.4). Samples should, therefore, include an adequate representation of the diverse entities of hematological malignancies routinely encountered in clinical testing. Normal samples as well as samples with different SV classes should be incorporated to assure a comprehensive assessment of assay performance. More specifically, all variant classes including CNAs, aneuploidies, balanced and unbalanced SVs (translocations, inversions, and insertions) should be evaluated. For subsequent additional validation(s), for example, addition of supplemental tissue types or additional diagnoses, a limited number of additional samples is usually sufficient depending on the complexity of the intended application.

The specimen types to be validated should include all the different sample types that will be encountered in routine practice. Peripheral blood and bone marrow specimens are the two most common sample types analyzed in cytogenetic laboratories testing hematological malignancies and there are established and supported OGM protocols for processing these two specific sample types (see Table 1). Other types of cells or tissues including, but not limited to CD-138^{+} enriched cell suspensions, fresh or frozen tumor tissue samples (e.g., myeloid sarcoma) are also amenable to analysis by OGM (Sahajpal et al.[21]). Furthermore, for each sample type, the sample collection and storage conditions must be clearly defined. Although remnant material from clinical specimens is the preferred sample for

validation studies, cell lines may also be used (e.g., for demonstrating the capacity of OGM to detect different SV variant classes or to demonstrate the detection of rare SVs). It should be noted that OGM is not validated for use on cytogenetic fixed pellets and cannot currently be performed on formalin fixed specimens, or from DNA previously extracted using conventional methods.

2.3.2 | Expected test performance

The expected performance should be defined upfront (before the start of the validation) and a sensitivity, specificity, precision, and accuracy of >90% compared to SOC methods (CBA, FISH, and/or CMA) is recommended. The test performances are calculated as shown in Table 1.

The calculation of performance metrics can be challenging when comparing technologies that have different resolutions (e.g., karyotype vs. OGM) or represent SVs only as copy number data (e.g., CMA vs. OGM). Therefore, as differences in size and breakpoint estimation may occur between OGM and orthogonal techniques based on the probe/label density at a given genome position, a variant can be considered concordant even if the size or breakpoint is slightly different. Furthermore, the resolution of OGM is considerably higher than CBA and, therefore, the detection of previously undetected abnormalities with OGM is expected. In general, we propose the following strategies to calculate sensitivity and specificity with SOC techniques:

1. OGM results should be compared with SOC results and only the reported aberrations with SOC methods should be used to calculate the true positive rate. Likewise, samples with normal results by SOC techniques should be used to calculate false positive and true negative events. Since normal genomes may harbor several polymorphic SVs, only clinically reportable SVs should be counted in control samples and these should be defined prior to the experiment. When calculating performance metrics, we recommend excluding polymorphic variants (SVs that are found in >1% of control population) and including only oncogenic SVs (e.g., Tier 1 SVs listed in the WHO, ICC guidelines for hematological neoplasms).[4–6] OGM performance evaluating large structural rearrangements including translocations, large inversions, and CNAs (≥5 Mb that should be visible by CBA; see Section 4) should also be part of the metric performance calculations. If a clinically reportable SV is detected by OGM in a cytogenetically "normal" sample, the laboratory should perform additional testing to confirm the presence of the SV that escaped detection by SOC testing. SVs that cannot be confirmed by orthogonal methods should count as false positives. Special care should be taken with abnormalities detected by one SOC technique that are at the LLOD for that technique. For example, a karyotype abnormality in two metaphases that cannot be confirmed by FISH or detected by OGM should be considered a false positive finding for karyotype rather than a false negative for FISH/OGM.
2. Another approach for calculating performance metrics would be to define hallmark SVs (for example, recurrent genetic abnormalities in AML) that would be assessed to measure the metrics. Each sample can then be compared for the "defined SVs" detected by the SOC method(s) and OGM. Cases where "defined SV's" are not observed by any of the technologies are counted as true negatives, while cases where "defined SVs" are detected both by SOC technologies and OGM are considered true positives. "Defined SVs" detected with SOC methods that are not detected with OGM are counted as false negative, while "defined SVs" detected with OGM that are not detected with SOC methods would require orthogonal confirmation with an alternate method in order to be counted as true positive (where possible). If an OGM-detected "defined SVs" remains unconfirmed, it would be counted as a false positive. "Defined SVs" that are detected by OGM only and subsequently confirmed, would be counted toward additional findings and would help affirm the additional clinical utility of the platform.

2.3.3 | Reproducibility

Reproducibility can be evaluated by performing intra-run, inter-run, inter-instrument, and inter-technologist comparisons. Laboratories will need to decide, based on local guidelines, how thoroughly reproducibility needs to be demonstrated. A thorough demonstration of reproducibility would include a sample run in duplicate/triplicate on the same chip (intra-run reproducibility) and on different chips (inter-run reproducibility). In laboratories with multiple instruments samples can be run parallel (inter-instrument reproducibility) to demonstrate the reproducibility of OGM across different instruments (also to validate new equipment). For sites with only one instrument, sample exchanges with other sites can be performed and can also serve as alternate proficiency testing.[22] Please note that some variability may be seen in small SV calls, especially near the limit of detection, as in some runs, they may meet the threshold for detection and in others not. However, larger SVs and those with higher variant allele fraction (VAF)/molecule counts should be consistently detected, excluding artefactual alignments that occur from time to time. The reproducibility should be measured for both technical (QC metrics) and analytical (clinically reported variant) performance. See Sahajpal et al.[21] for a comprehensive demonstration of reproducibility.

2.3.4 | Limit of detection

The LLOD should be assessed for different variant classes, which can be done empirically or *in silico*. For empirical measurement, the experiment can be done at the cell or DNA level. The cells from a sample with a known variant and known allele fraction can be mixed with wild-type cells to achieve a dilution series that can be used to test the LLOD (e.g., 50%, 25%, 10%, 5%, and 1%). As an alternative to using cells, DNA can be mixed to yield the required allele fraction dilutions.[21] For *in silico* LLOD determination, the laboratory can use

two samples, one with several abnormalities (preferably with different SV classes) and a normal control, and then down sample the molecule files (.bnx) in different proportions to represent the various "dilutions."[19] This process will demonstrate the limits of the assembly pipeline to detect low-level events. Please note that the LLOD is also dependent on variables such as quality of DNA (N50 >150 kb, map rate, coverage, CNA-tool/SV-tool), and coverage (see Section 3 for further detail).

2.4 | Clinical validation

Concordance can be measured as the fraction of abnormalities detected with both tests (SOC and OGM) or as the fraction of patients in which all abnormalities detected by SOC tests are also detected by OGM. Concordance should be evaluated for all clinically relevant abnormalities detected using SOC methods. To calculate diagnostic sensitivity, the diagnostic yield of OGM should be compared to the diagnostic yield of the old SOC test. Diagnostic yield can be measured as the fraction of patients with a clinical condition who have an abnormal result for the relevant laboratory test. We recommend that only clinically relevant abnormalities are included in the comparison.

When calculating concordance and diagnostic yield, a representative cohort of samples should be used to compare the clinically relevant abnormal results for each subtype of hematological malignancies (according to WHO and/or ICC). It is important to also include cases reported as normal by SOC methods. As previously mentioned, the validation cohort size depends on the type of hematological malignancy to be validated. For hematological malignancies with only a small number of hallmark cytogenetic abnormalities (e.g., CML) or for region specific testing a smaller cohort is likely sufficient (e.g., $n = 10$). However, if the clinical validation represents several hematological disorders associated with a diverse spectrum of cytogenetic abnormalities (e.g., myeloid neoplasms), a larger sample cohort is required (we recommend at least 30 samples). Note that samples used for technical validation can be used again for the clinical validation(s).

For example, a recent clinical validation study for AML, performed by one group in this consortium, used 42 samples (20 of which were found to have clinically relevant abnormalities by SOC testing). OGM yielded the same result for 41/42 cases thus demonstrating a concordance rate of 97.6% (based on the total number of patients analyzed). SOC testing identified 91 clinically relevant cytogenetic abnormalities in the 42 AML patients of which 90 were detected by OGM. Thus, the concordance based on the number of cytogenetic abnormalities is 98.9%. The diagnostic yield of SOC testing was 47.6% (20 abnormal samples out of 42). OGM uncovered clinically relevant abnormal results in 23 of the 42 samples, resulting in a diagnostic yield of 54.7%. The increased diagnostic yield of OGM over SOC testing in this specific clinical validation study of AML patients using a cohort of 42 patients was, therefore, 7.1%. In addition to concordance and diagnostic yield, other parameters such as success rate, turn-around time, and cost should be included to document the impact that OGM has on the overall laboratory process as well as to assure the clinical benefits for the patient.[23]

2.5 | Implementation

Prior to clinical implementation of OGM, it is important to finalize your standard operating procedure documents, establish a training and competency plan for laboratory staff, develop a standardized interpretation and reporting approach (see Section 4) and have a set of report templates that conform to local and international regulatory requirements (e.g., College of American Pathologists). Implementation of a new laboratory test may have implications for other work processes and logistics within the laboratory. Therefore, a chain test can be performed to verify minimal/negligible impact of OGM on laboratory operations. A chain test is an end-to-end test during which the entire chain of systems from the first input to the last output is tested by running one or more samples through the entire route before the new test is implemented. A risk inventory is also a valuable step prior to implementation where one considers all the potential risks with a technique (e.g., equipment failure, server/IT failure, human resources, etc.), the likelihood of their occurrence and a plan to deal with them if they occur (e.g., downtime procedures). A risk inventory is also critical for staff training considerations.

3 | QUALITY CONTROL CONSIDERATIONS

Data quality is crucial for OGM as the ability to assemble meaningful maps depends heavily on the length of intact DNA molecules and the fidelity of the labeling. Accordingly, sufficient emphasis should be given to ensure the best possible QC values, and all material/tissues should be stored in appropriate conditions to preserve cell membrane integrity for sample prep.

3.1 | Samples

Peripheral blood or bone marrow aspirate samples for OGM analysis should preferably be collected in EDTA tubes. Samples collected in sodium heparin tubes should be spiked with DNA Stabilizer as soon as possible, as heparin alone is a poor stabilizer for OGM applications (https://bionano.com/wp-content/uploads/2023/01/30358-Bone-Marrow-Aspirate-Shipping-Instructions-3.pdf). Samples should be processed as expediently as possible to ensure the extraction of high quality UHMW DNA. Samples in vacutainers can be kept for several days at 4°C and for longer storage, should be frozen at −80°C. Whenever possible, at least two aliquots should be prepared for storage. This safeguard allows DNA extraction from the original sample to be repeated should a quality or reagent problem arise during processing of the first aliquot. Subsequent handling of samples, for example, during isolation and labeling, should be done as carefully as possible to prevent DNA shearing during sample preparation.

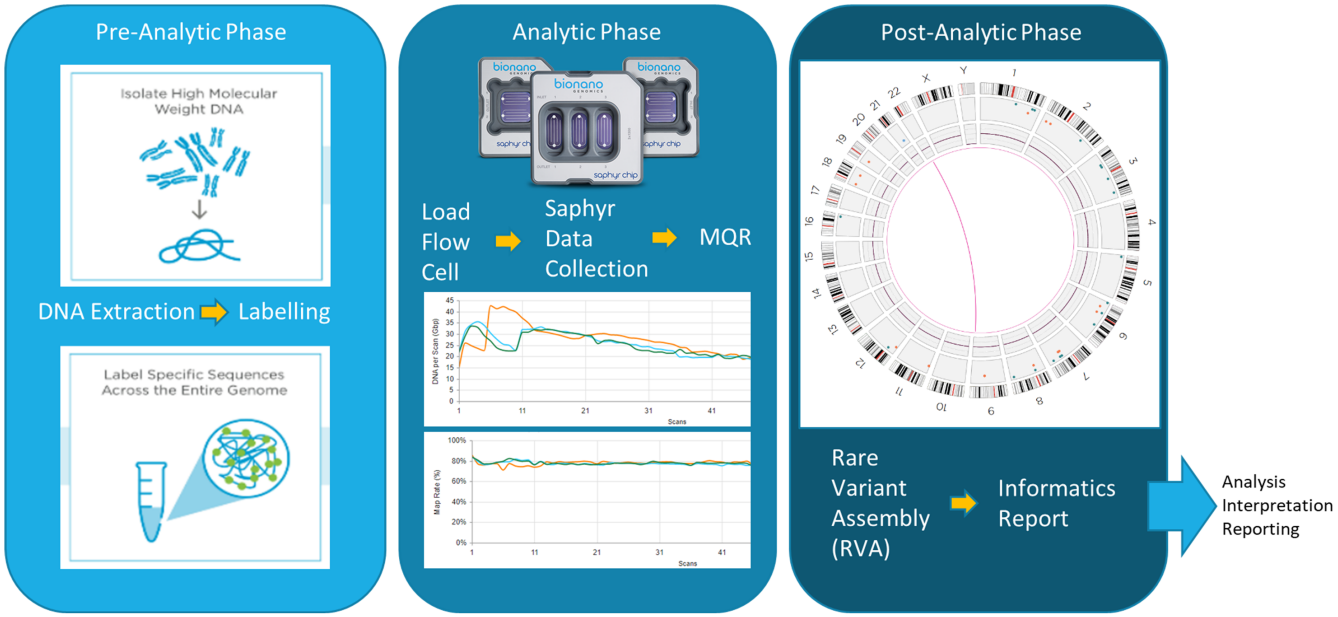

FIGURE 1 Schematic diagram illustrating the components in each of the three phases of performing OGM. The pre-analytical phase involves sample preparation, DNA extraction, and labeling. See Table 2 for relevant details. The analytical phase involves loading the labeled sample onto the flow cells and collecting the data from a sample. See Table 3 for relevant quality metric information that is contained in the MQR. Samples should have sufficient quality before proceeding to the post-analytical phase. In the Post-Analytical Phase a bioinformatics genome assembly is performed (rare variant assembly for somatic analysis). Several critical quality metrics that are generated here are summarized in Table 4. Once the data has been collected and is of sufficient quality, the sample can be analyzed and reported. OGM, Optical Genome Mapping; MQR, molecule quality report.

Solubilized DNA should never be pipetted harshly, vortexed, or frozen. Use of other types of samples, such as fresh/frozen tumor samples, cytological aspirations or other non-fixed specimens should be evaluated by the laboratory for suitability and optimal storage conditions.

At present, all sample preparation steps are performed manually, with protocols for automation currently in development. Good laboratory practice dictates that only one open DNA aliquot/tube should be processed at a time to minimize the risk of sample mix-ups and barcoding (e.g., using barcode stickers on microcentrifuge tubes) should be used wherever possible.

The following sections detail the pre-analytic, analytic, and post-analytic phases of the OGM assay. These phases, along with the critical QC steps, are summarized in Figure 1.

3.2 | Pre-analytical phase

3.2.1 | Cell input, DNA concentration, and DNA isolation

While the manufacturers recommend 1.5 million viable cells as the starting input material, published protocols indicate that a range between 1 and 1.5 million cells is acceptable (Table 2). Achieving an ideal input cell concentration is critical for in-range quality metrics downstream. "Low input" protocols are under development and may be indicated in certain scenarios where cells are limited (e.g., $CD138^{+}$ isolated cells in plasma cell neoplasms); however, it should be noted that lowering input may increase the incidence of sample failures due to inadequate DNA yield.

The DNA solution will be quite viscous following DNA isolation due to the UHMW DNA fragments extracted. The DNA solution is left overnight or for several days to homogenize depending on the number and quality of the input cells, and resulting DNA solution's electrostatic properties. It is generally easier to accurately assess cell input number from unfrozen samples (especially blood) and the extracted DNA usually homogenizes easily. The DNA solution following isolation from frozen bone marrow aspirates, especially cryopreserved cell pellets, typically take longer to homogenize and tend to have lower N50 values. This is primarily because these types of samples often have dead cells that generate degraded DNA and protein contamination. Several techniques can be used to improve the quality of UHMW DNA retrieved from samples with high numbers of dead cells including: (1) centrifugation of intact cells (https://bionano.com/wp-content/uploads/2023/01/TECHN-00001-Bionano-Prep-SP-Frozen-Cryopreserved-Cell-DNA-Isolation-Technote-2.pdf), (2) apoptotic cell selection kits (e.g., annexin V negative selection), or (3) flow cytometric or microfluidic sorting of live cells.

To assess whether the DNA is adequately homogenized, the DNA concentration is measured in three different regions of the solution and the corresponding coefficient of variation is calculated. A coefficient of variation ≤0.30 indicates that the DNA solution is sufficiently uniform and the mean of the three DNA concentration measurements can be considered accurate (Table 2). Once the DNA is within the ideal range (Table 2), 750 ng of DNA is used for the labeling reaction with the DLE-1 enzyme, which should produce a labeled DNA concentration between

4 and 16 ng/μL. The labeled DNA is quantified to ensure the optimal concentration has been obtained and then loaded into the flowcell.

3.3 | Analytic phase—Quality control parameters

Once samples have been loaded onto the flowcell and imaging begins, DNA per scan (Gb) and Map Rate (%) can be visualized in real time using the Saphyr Instrument Control Software and Bionano Access dashboard. From the dashboard, run time can be estimated as well as an initial assessment of sample quality. At this stage, low quality samples, poor labeling, clogging, or other problems with the flowcell can be identified by evaluating the real-time data collection (for more detailed information and troubleshooting see https://bionano.wpenginepowered.com/wp-content/uploads/2017/03/30304-Bionano-Access-Dashboard-Guidelines.pdf). Once data collection is complete (e.g., 1500 Gb for rare variant analysis [RVA]) a molecule quality report (MQR) can be reviewed to evaluate the quality metrics of the sample. Samples that do not meet ideal QC targets may still be analyzed but care should be taken in the interpretation as sensitivity for the detection of SVs and CNAs may be reduced (see below). Three main quality parameters should be assessed:

1. Molecule length (N50 of molecules ≥150 kb and N50 of molecules ≥20 kb). This metric evaluates the N50 length of the extracted and labeled DNA molecules. Long DNA molecules are essential for optimal alignment to the reference genome and for detection of SVs by the pipeline. Short molecules, which can result from sheared DNA either during sample preparation or from an initially degraded sample, will likely correspond to a lower map rate and a less sensitive analysis (Table 3).
2. Map Rate refers to the percent of molecules that are mapped to the reference genome. It is a useful proxy for the proportion of raw data that is expected to be used in downstream analysis. A map rate of greater than 70% is recommended.
3. Effective coverage is calculated from the total DNA collected (in Gb) multiplied by the map rate, divided by the size of the reference genome (e.g., human haploid genome ca. 3.1 Gb). For example, if 1500 Gb of DNA is collected and the map rate is 100% (1.0) for a human genome (3.1 Gb), the resulting effective theoretical maximum coverage would be ca. 484×. If the map rate is reduced to 70% the effective coverage drops to ca. 339×. High-effective coverage is essential for the detection of low allele frequency events and accurate detection of copy number changes.

TABLE 2 Recommended targets for cell input, DNA concentration, and post-labeling DNA concentration.

parameter	Target	Common reasons for missed target
Input sample: cell count	1 500 000 viable cells/sample	• Improper sample handling, storage, stabilization • Low sample volume availability or paucicellular sample
DNA concentration	39–150 ng/μL	• Inaccurate cell input during DNA isolation • Excessive DNA mass loss during isolation related to inhibitory substances in lysate and/or fragmented DNA: ○ DNA mass fails to precipitate from lysate ○ DNA mass detaches from nanobind disk
DNA conc. coefficient of variation (CV) among three replicate measures $CV = \frac{\text{standard deviation}}{\text{mean}}$	≤0.30	• Isolated DNA needs more time and/or gentle mixing to homogenize • DNA is too concentrated
Labeled DNA concentration	4–16 ng/μL	• Inaccurate quantitation of input DNA • Low labeled DNA recovery from Direct Label and Stain (DLS) membrane

3.3.1 | Bioinformatic assembly pipelines

If a molecule file (.bnx format) is considered to be of sufficient quality, it can be analyzed using either the de novo assembly (used mostly for

TABLE 3 Analytical quality metrics–the molecule quality report.

Parameter	Target	Common reasons for missed target
Effective coverage	≥340×	Effective coverage $= \frac{\text{total DNA} \times [\text{map rate}]}{\text{reference size}}$ So, • Inadequate total DNA in the data set • Low map rate (<70%)
N50 (≥150 kbp and minimum labels ≥9)	≥230 kb	• Deteriorated cell membrane integrity/DNA length from original sample • Excessive DNA shearing during sample prep or storage
N50 (≥20 kbp)	≥150 kb	
Map rate	≥70%	• Low label density/poor labeling efficiency • Short DNA molecules • DNA becoming stuck in the nanochannels

TABLE 4 Post-analytic quality metrics and troubleshooting–Informatics report.

Parameter	Target	Common reasons for missed target
Sex	Consistent with indication	• Sex chromosome abnormalities could confound X/Y sex determination • Medical (e.g., transplantation) history may confound X/Y sex determination
Effective coverage of reference	≥300×	Effective coverage of reference $(X) = \frac{\text{total DNA aligned to the reference in pipeline}}{\text{reference size}}$ • Inadequate total DNA in the data set • Low map rate (<70%) • Poor analytical QC generally
CNV statistics: percent above expected (2 Mbp/6 Mbp window)	≤+20	• Poor analytical QC generally • Poor run performance • Poor label clean-up in DLS procedure • Expired or improperly stored Proteinase K used in DLS procedure
CNV statistics: correlation with label density	≤0.25	

germline/constitutional analysis) or the RVA (for somatic analysis). This framework is focused on hematological malignancies and will thus discuss only the RVA in detail. The de novo assembly may be useful in some situations (e.g., for the detection of very small SVs where the lower size limit is 500 bp vs. 5 kb for the RVA, or detection of loss of heterozygosity [LOH]). Clearly, the de novo assembly would be the tool of choice for evaluating abnormalities that are suspected to be constitutional or also for the evaluation of potentially hereditary SVs that may contribute to leukemic predisposition. There is growing recognition that structural variants contribute to cancer predisposition and are likely under ascertained by current NGS-based approaches.[24,25] However, these applications are outside the scope of this framework.

In contrast to the de novo assembly, the RVA detects structural variants at low VAF—5% at 300× coverage (https://bionano.com/wp-content/uploads/2023/01/30110_Rev.L_Bionano-Solve-Theory-of-Operation-Structural-Variant-Calling.pdf).[21] Accordingly, it is the pipeline of choice for the analysis of cancer genomes. Unlike the de novo assembly, which generates the patient/sample genome first and then calls variants by comparison to a reference genome, the RVA works without a whole genome assembly. Instead, the single molecules are directly compared to the reference using a split read analysis and a copy number analysis. The split read analysis looks for clusters of molecules with internal alignment gaps or multiple alignments, requiring only three molecules for an SV to be called. The copy number analysis highlights regions with increased or reduced coverage. The RVA allows for the detection of insertions of 5–50 kb, deletions ≥7 kb, translocations (or transpositions) ≥70 kb, inversions ≥100 kb and duplications ≥150 kb, while the de novo assembly tool can detect SVs down to a 500 bp resolution but is not designed for VAFs below 20%–25% (https://bionano.com/wp-content/uploads/2023/01/30110_Rev.L_Bionano-Solve-Theory-of-Operation-Structural-Variant-Calling.pdf).

3.4 | Post-analytic quality parameters

Samples that have completed an analysis pipeline will generate an informatics report that can be used to evaluate the limitations of the final assembly analysis and determine if the data meets the quality metrics and/or criteria established by your laboratory for reporting (Table 4). Samples should have an effective coverage of ≥300× for high sensitivity of somatic SVs down to a 5% VAF, and CNAs down to 10%–15% VAF. Analyses with effective coverage lower than 300× are informative, though may have reduced sensitivity to the lowest VAF events.

The copy number output produces a copy number profile that uses depth of molecule alignment coverage to call copy number gain and loss segments. The informatics report contains a copy number variant (CNV) statistics section, which summarizes certain signal and noise attributes of the copy number profile. Occasionally samples with non-ideal analytical QC will produce noisy copy number profiles, evidenced by CNV statistic parameters that exceed critical values (Table 4). These profiles tend toward higher amplitude waves in the CNV profile, can have difficulty normalizing a copy number baseline, and show systematically high copy-neutral (CN) gain/loss calls as an artifact.

When assessing sample quality metrics, special attention should be paid to the effective coverage of reference from the assembly informatics report. Effective coverage of the reference is estimated during and after the data collection step, and after the RVA is complete. The effective coverage of the reference is expected to drop between the two steps as the second measure of effective coverage is generated from only the molecules that are included in the assembly. Lower effective coverages will likely reduce the sensitivity for the detection of low allelic fraction events.

3.4.1 | Sub-optimal analyses

In silico analysis indicates that samples that meet the quality metrics shown in Table 3 will likely result in a genome assembly with adequate post-analytic quality metrics. The relationship between effective coverage of the reference, SV, CNA, and aneuploidy sensitivity is described in detail here: https://bionano.wpenginepowered.com/wp-content/uploads/2022/05/30110_Rev.L_Bionano-Solve-Theory-of-Operation-Structural-Variant-Calling.pdf. The lower limit value indicating reduced sensitivity for the detection of structural and CNAs for other analytical QC metrics like N50 and map rate have not yet been empirically determined. This work is in progress and will likely be the result of

TABLE 5 Suggested reporting comments for samples with quality issues.

Category	Description of quality scenario	Final report comment
Failed sample	DNA too low concentration or too poor to label—not run on flowcell	The DNA was of insufficient quality to perform Optical Genome Mapping. Repeat testing on another samples is recommended, if clinically indicated
Failed assembly	An assembly is generated but QC metrics too poor for confident analysis—often with >2 analytic or post-analytic metrics below target (both >10%)	The result for this Optical Genome Mapping analysis is considered a failure. Multiple sample and assembly quality metrics were below the recommended thresholds resulting in an analysis with low confidence and sensitivity. This may be caused by a variety of technical and biological factors. Repeat testing on another sample is recommended, if clinically indicated
Sub-optimal result (multiple factors outside target or >10% below target)[a]	>1 quality parameter outside recommended range (either analytic or post-analytic, but within 10%) or 1 factor outside 10% tolerance)	Optical Genome Mapping was performed on this sample; however, the molecule quality was sub-optimal. Sub-optimal samples may have reduced sensitivity for the detection of structural and copy number abnormalities (CNAs). This result should be interpreted with appropriate caution
Near-optimal result (within 10% tolerance)[a]	1 quality parameter within 10% tolerance—either analytic or post analytic)	Optical Genome Mapping was performed on this sample; however, (N50/map rate/effective coverage/CNV statistic) was below the established target but within 10% of the target value. This will likely not impact the quality of this analysis however near-optimal samples may have slightly reduced sensitivity for the detection of structural and CNAs. This result should be interpreted with appropriate caution

[a]Referring principally to analytic QC factors (1) molecule N50 >150 kb, (2) effective coverage, or (3) map rate or post-analytic QC factors (1) effective coverage or (2) CNV statistic.

a separate publication or white paper that will provide additional guidance and empirical/*in silico* evidence where the limits of detection are severely affected. However, it should be noted that multiple assay and sample problems can lead to quality issues on the MQR. In general, this group's experience has shown that a 10% variance below the target for any one of molecule N50, Map Rate or Effective Coverage will likely not significantly impact the quality of the analysis. However, if not all the molecule quality targets are met, we recommend indicating as such on the clinical report and commenting that the analysis is "suboptimal" and should be interpreted with appropriate caution (see Section 3.4.2). For troubleshooting of assay problems, we refer to Bionano's documentation: https://bionano.com/wp-content/uploads/2023/01/CG-30608_Rev.A_Bionano-SP-G2-and-DLS-G2-Troubleshooting-Guide-1.pdf.

3.4.2 | Low quality samples, clinical necessity, and irreplaceable samples

In clinical practice, a laboratory often receives biological samples that are sub-optimal for testing. Common reasons include: insufficient sample volume, excessive time in transit, paucicellular samples, dry taps or other biological or technical factors that are known to impact DNA molecule quality. Given that these samples are precious and may be irreplaceable, every attempt should be made to generate a clinical result that could help direct patient care. The failure of multiple quality metrics indicates that the analysis may have low sensitivity for the detection of clinically relevant SVs and may also have noisy copy number data resulting in false positive calls. We present some general guidelines and recommendations in Table 5 for reporting results derived from patient samples of low quality. Ultimately, the decision to report results on poor-quality samples lies with each individual laboratory.

4 | DATA EVALUATION, INTERPRETATION, AND REPORTING

4.1 | General considerations

As OGM is a DNA-based analysis, the approach for the interpretation of the clinical significance of detected variants has largely been adapted from the recommendations developed by professional organizations for somatic sequence variants,[26] as well as CNAs and CN loss-of-heterozygosity (CN-LOH) detected by CMA.[27,28] The long-term goal is to generate a "technology agnostic

classification"[29] and maintain a consistent framework for interpretation across techniques.

There are important differences between the data obtained from OGM relative to karyotype analysis and CMA that are pertinent to the interpretation and reporting of variants. Many of the relevant differences between OGM and CBA are detailed in the introduction. However, OGM is a bulk genome technique (like NGS) and a karyotype-style "cell-level" report is replaced by an interpretation and nomenclature report more aligned with CMA. In contrast to CMA, OGM detects both balanced rearrangements and CNAs. Correlation and integrated interpretation of structural and CNAs are required.

4.2 | Using the rare variant assembly

For analysis of cancer samples, the consensus recommendation is to use the RVA as the primary analysis pipeline. The de novo assembly may be useful in certain specific disease scenarios but is beyond the scope of this framework. The RVA provides both a combination of structural variants and CNAs. When reviewing data, it is important to recognize that the same abnormality can be identified by both an SV call and a copy number call. For example, a large interstitial deletion on a chromosome will be recognized by the SV algorithm as an intrachromosomal fusion but will usually also show a copy number loss. Therefore, the two data types must always be interpreted together, and a consensus SV reported based on the evidence.

For the RVA, all variants involving cancer-associated genes should be reviewed. Region files of specific sub/classes of the hematologic malignancy (in BED format) may also be compiled and used in the review. The proposed cut-off for RVA at 5 kb is applicable. Reviewing of variants outside of these recommended thresholds should be performed only at the discretion of the individual laboratory. However, these smaller variants of possible clinical significance based on literature evidence of clinical trial data would need orthogonal confirmation.

4.3 | Phases of analysis

When beginning analysis on a new sample, the filter settings should always be reviewed and reset. This is accomplished by opening the filter settings dialogue and pressing "Reset Filters" (to their default values). While there are many potential filter setting variations that can be used for analysis, based on our collective experience, we recommend the following settings (Table 6).

We recommend using the "ALL STRUCTURAL VARIANTS" and "ALL COPY NUMBER VARIANTS" with a 1% control database threshold rather than only viewing SVs using the NON-MASKED VARIANTS option. While this may slightly increase the number of variants that need to be evaluated, the likelihood of detecting a rearrangement that overlaps a masked region is improved and non-relevant SVs are usually easily discarded from the analysis upon manual inspection (see below, guidelines for manual variant inspection).

TABLE 6 Recommended filter settings for analysis.

Filter section	Filter parameters	Recommended setting
SV type	Insertion, deletion, inversion, duplication, intra-fusion, inter-translocation	Recommended
General SV filters	SV masking filter VAF filter min VAF filter max	All structural variants 0 1
Variant annotation filters	SV in ≤ this % in control db with the same enzyme SV self-molecule check Self-molecule count SV in ≤ to this % of the control db SV overlapping genes filter	1%[a] SV found in self molecules 5 1%[a] All SVs
Copy number filters	Copy number variant type Copy number variant confidence Copy number variant minimum sizes (bp) Copy number variant masking filter	All Recommended 500 000 All copy number variants
Aneuploidy filters	Aneuploidy type Aneuploidy confidence	All Recommended

Abbreviations: SV, structural variation; VAF, variant allele fraction.
[a]This value can range from 0% to 2%. Laboratories should evaluate how changing this value, changes the data for their specific applications.

4.4 | Criteria for variant review

Manual review of the filtered variants is recommended to identify SV calls that meet established criteria, are supported by molecule data and, to eliminate potential artifacts. The RVA groups molecules with similar label patterns and attempts to map them back to the reference. Erroneous unbalanced SV calls (e.g., insertions, deletions, duplications, and translocations) can occur because of poor alignment due to N-base gaps in the reference genome, blocks of segmental duplications, or large regions of repetitive sequence that are present in many locations throughout the genome (e.g., transposons). In addition, alignment of molecules in genomic regions around the centromeres and telomeres may lead to variability in coverage and artefactual can calls often due to the highly repetitive nature of these sequences and highly similar blocks of segmental duplications present around the centromere of several chromosomes (e.g., chromosome 1 and 9 being prototypic examples). These artifacts are generally straightforward to identify and distinguish from the true clinically relevant SVs identified by OGM. The guidelines and points to consider below will facilitate identification of the majority of clinically relevant SVs and eliminate artefactual calls.

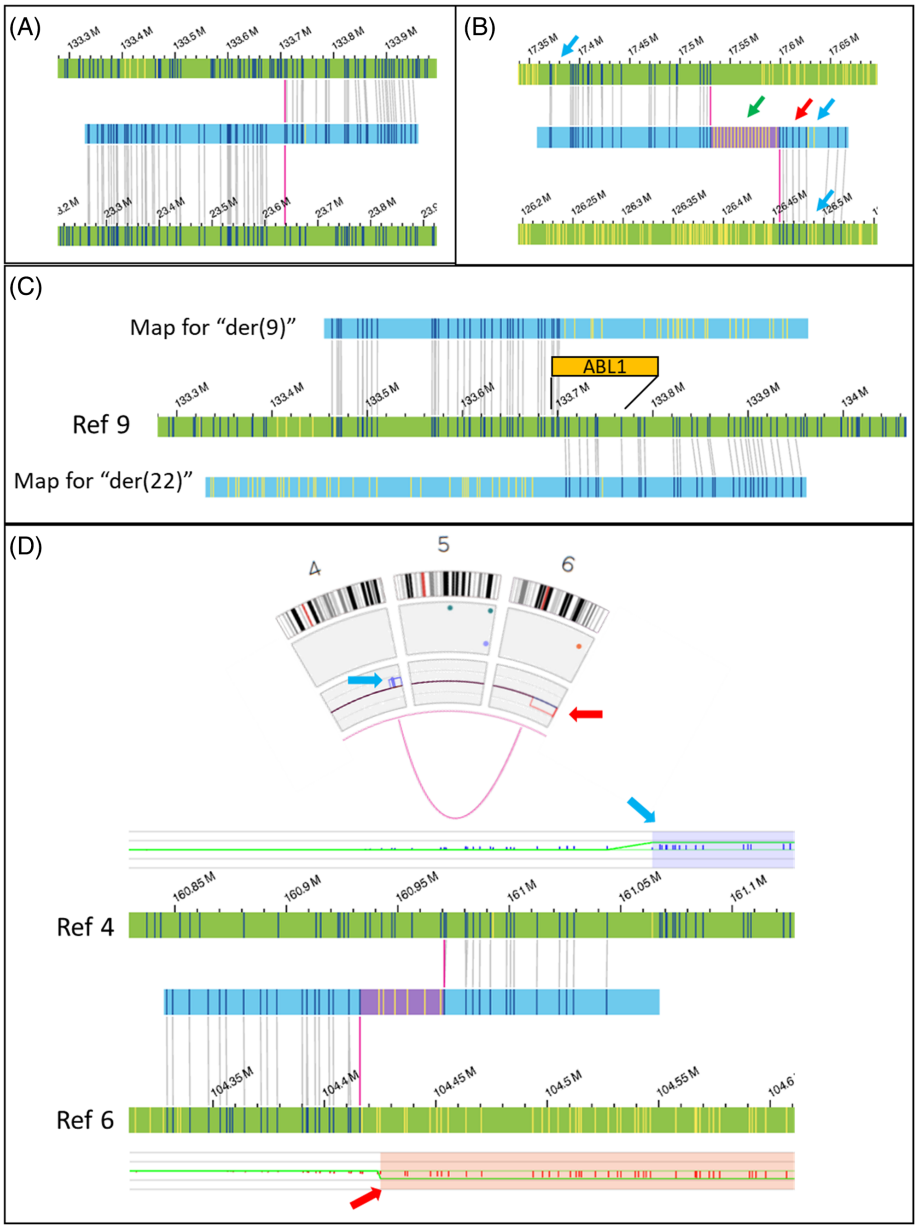

FIGURE 2 Evaluating structural variation (SV) and CNA calls in Optical Genome Mapping data. (A) Interchromosomal translocation supported by >10 labels on each side of the breakpoint (magenta vertical line) and with 85 supporting molecules and confidence of 1 (not shown, from SV data). (B) Interchromosomal translocation with <10 (poor) label support on the right side of the breakpoint (red arrow) with 155 supporting molecules but a confidence of only 0.13 (not shown, from SV data). Other elements that are frequently seen in artefactual calls may include labels present in the reference and not present in the hybrid map, or vice versa (blue arrows show several examples). It should be noted that polymorphisms between samples and the reference may have labels that do not always correspond or align, however, seeing multiple label variances is an indicator of a poor-quality alignment. Looking for repetitive label sequences on both reference chromosomes may also suggest the alignment is a result of similar label patterns on both regions of the reference where the SV call is. Repetitive sequences may also appear with different lengths in the sample versus the reference (green arrow) due to individual heteromorphisms. (C) Reference chromosome 9 is displayed (green bar) with two hybrid maps representing a balanced *BCR::ABL1* translocation. In Genome View by selecting "Anchor 9" and "Show All" in the region of the translocation, both hybrid maps can be visualized simultaneously. The top map (blue bar, "der(9)") aligns proximally to Ref 9 (gray matchlines with blue labels) and does not align to Ref 9 distally (no matchlines and labels are yellow) since this part of the hybrid map align to chromosome 22. This is the equivalent of the derivative 9 since it is connected to the 9 centromere. Conversely, the der(22) hybrid map shows yellow labels with no matchlines on the left side as it aligns to chromosome 22, but at the breakpoint, the matchlines appear and labels turn blue showing the hybrid map is now aligning to the more distal segment of Ref 9 shown. The presence of both hybrid maps is good evidence of a reciprocal translocation. Note that breakpoint locations may differ between derivatives and the presence of segments of deleted sequence between breakpoints is not uncommon. (D) An excerpted Circos plot showing only chromosomes 4, 5, and 6 shows an interchromosomal fusion call between chromosomes 4 and 6 (magenta line) and also associated copy number gains on chromosome 4q (blue lines) and copy number losses on chromosome 6q (red lines). In the Genome View below the Circos plot, the unbalanced hybrid map is shown with the copy number tracks above (Ref 4) and below (Ref 6) showing a gain for chromosome 4q and a loss for chromosome 6q.

4.4.1 | Intra- and inter-chromosomal fusions (i.e., translocation, large insertions, and inversions)

1. SV calls observed at 5% or greater allele burden, supported by ≥10 molecules with at least 10 labels on either side of breakpoint provide strong evidence for a true positive call (Figure 2A).
2. True positive calls often have high confidence scores. However, confidence modeling in the Bionano Solve pipeline was developed based on models using simulated data and may not always capture the range of complexities in real cancer genomes (Bionano Solve Theory of Operation: Structural Variant Calling, https://bionano.com/wp-content/uploads/2023/01/30110_Rev.L_Bionano-Solve-Theory-of-Operation-Structural-Variant-Calling.pdf). True rearrangements in genomically repetitive or complex regions—especially near regions with low DLE-1 site densities or uniqueness (centromeres, terminal ends of chromosomes)—may feature poorer map alignment and be constrained to lower confidence scores (Figure 2B). Therefore, we recommend using confidence scores as a starting point for considering true calls, among other criteria. SV calls that meet other thresholds such as VAF >5%, >10 molecules, and >10 labels on each side of the breakpoint, but with low confidence can be reported—although these findings may require orthogonal confirmation in some situations.
3. Variant calls noted at <5% VAF or supported by fewer molecules (between 5 and 10) would need confirmation using an orthogonal platform before reporting. If the variant call is deemed clinically significant and confirmation is not possible, the laboratory may opt to report the variant with a comment explicitly stating the weak molecule support for that particular variant and the need for confirmation using an alternate platform. An important consideration is the variable proportion of cancer cells within any sample. This is especially pertinent in cases with low cancer cell fractions where true SVs are present but have low VAFs and thus reduced molecule support. In all cases, the results must be correlated with the clinicopathological features.
4. SV calls that are artifacts may occur with relatively high VAFs and have greater than 10 molecules supporting the call. These calls often have 10 or fewer labels that align on one side of the breakpoint (Figure 2B). In addition, hybrid maps (blue bars) with <10 labels often have labels that are not in the reference or labels in the reference that are not in the hybrid map. This label variance is another strong indicator of an artefactual call. SV calls with less than 10 aligned labels should be interpreted cautiously. By activating the DLE-1 SV Mask track, it is possible to see regions where artefactual calls are more common, such as N-base gaps in the reference sequence (a region of unknown length), common repetitive elements or regions with segmental duplications. Calls that overlap DLE-1 mask regions with poor label support are almost always artifacts.
5. It is important to determine if an inter-chromosomal rearrangement is balanced or unbalanced. Balanced rearrangements will have two hybrid molecules (one for each derivative) and no loss or gain of chromosomal material around the breakpoint (Figure 2C). Unbalanced rearrangements will often only show one hybrid map and should have copy number gains or losses at the breakpoint of the involved chromosomes (Figure 2D). If an inter-chromosomal rearrangement is observed with only one hybrid molecule AND with no copy number loss or gain—this is a strong indicator that the SV call may not be real. It should be noted that due to the slightly different analytic sensitivity of the SV and CNA calling algorithms when an SV is present near the LLOD, the associated copy number change may not be called with recommended filter settings (Figure 3A). A careful inspection of the whole genome copy number view is recommended as well as careful consideration of whether to include the SV in the final report.

4.4.2 | Unbalanced SVs called by the SV algorithm (i.e., deletions, duplications and insertions <500 kb)

These calls are generally easier to interpret as they do not involve the joining of two distant chromosomal regions. As such, the variant calls usually demonstrate a loss of labels and/or a loss of inter-label distance between two well-aligned flanking labels (a deletion), a duplicated set of labels directly adjacent to one another (direct or inverted duplication) or an increase of inter-label distance intervening in a sequence, often with several unmapped labels (unidentified insertion). Note that small duplications with low numbers of labels may be called as insertions, such as the KMT2A partial tandem duplication, as sufficient duplicated sequence with corresponding label pattern is not present to identify the SV unequivocally as a duplication. Larger insertions, where sufficient labels are present to identify the donor and recipient regions should be evaluated as detailed in the section above (Intra- and Inter-chromosomal Fusions).

1. SV calls observed at 5% or greater allele burden, supported by ≥10 molecules are likely real.
2. The SV confidence score may be used, keeping in mind that the confidence score can be variable depending on the genome context (see above). Importantly, confidence scores are not calculated for duplications and always show "−1," and the confidence for deletion and insertions is set to "0" in the recommended filter settings.
3. False positive calls may occur in reference regions featuring segmental duplications and/or palindromic sequences. SV calling may extrapolate deletions/duplications between low-copy repeats. This could occur, for example, if the alignment process pairs non-unique map segments to an incorrect low-copy repeat further away. In certain scenarios, artefactual inversions or inverted duplications can occur in palindromic reference regions due to orientation challenges in the molecules to map assembly.

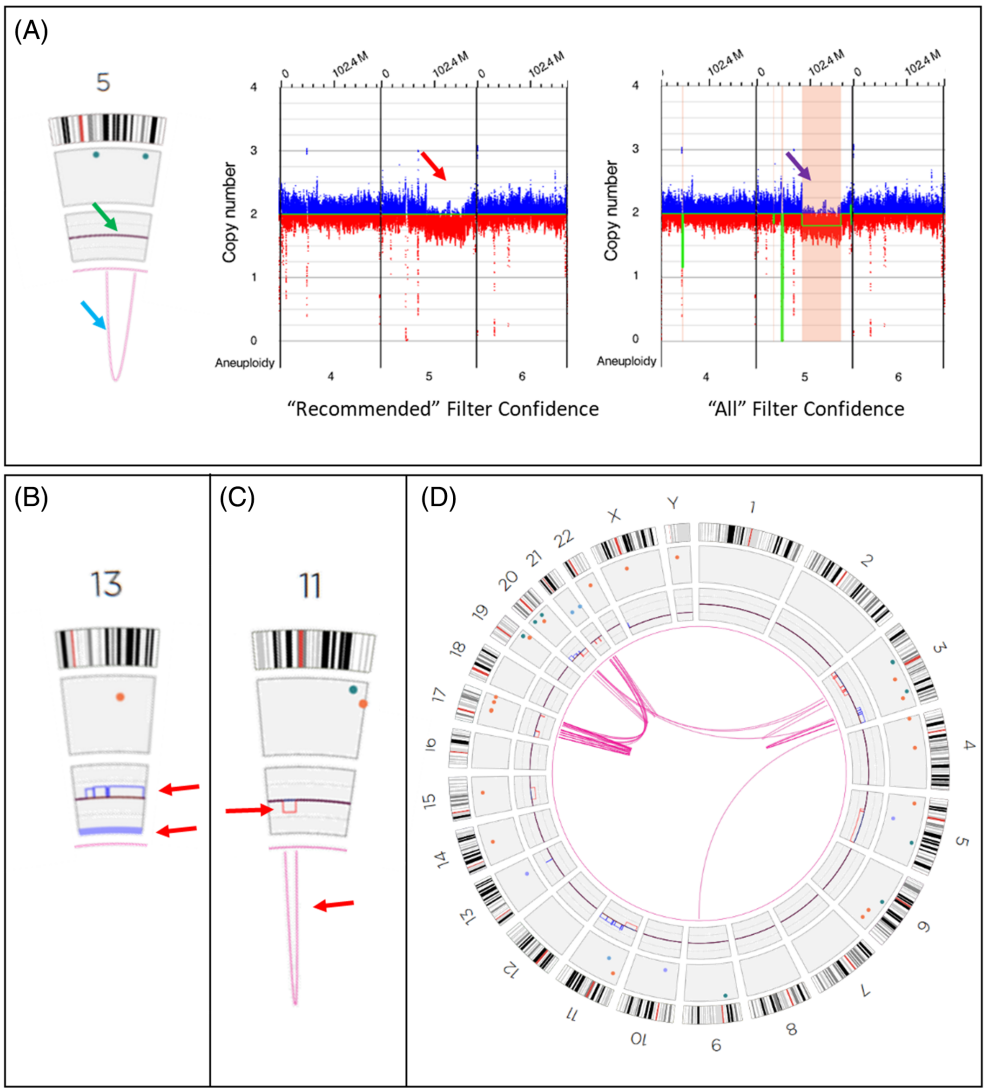

FIGURE 3 Synthesizing structural variation (SV) and copy number calls and adjusting filter settings. (A) A concurrent bone marrow sample from this patient showed a deletion of chromosome 5q. A peripheral blood sample was tested for Optical Genome Mapping with a much lower proportion of abnormal cells. The SV caller detected the intrachromosomal fusion of the deletion segments on chromosome 5q; however, the copy number was at the threshold for the copy number algorithm using the "Recommended" Filter setting. However, a manual inspection of the copy number data in the genome view shows a clear deflection in the copy number track corresponding with the breakpoints of the SV call (red arrow). When the copy number filter settings are changed to "ALL" the 5q deletion is called (purple arrow). Visual inspection of copy number data is highly recommended. (B) A sample showing trisomy for chromosome 13. The copy number track shows several segments of increased copy number (top red arrow) and the bottom blue line showing the chromosome aneuploidy call (bottom red arrow). Note that the p-arms of acrocentric chromosomes are masked due to their repetitiveness. There is no SV call accompanying this copy number call, since it is whole chromosome. (C) An interstitial deletion on chromosome 11p. Note the copy number loss called on the copy number track (top red arrow) and the SV call (bottom red arrow) indicating the fusion of distal and proximal breakpoints of the deletion. (D) A circos plot of a sample with a complex genome. There are several notable findings: (1) chromoplexy involving chromosomes 3, 17, 20 and 21, (2) chromosome 5 and 15 are involved in an unbalanced rearrangement involving the chromosome 5 centromere (therefore, no SV call, but observed by concurrent karyotyping); however, the exact copy number changes are visible (i.e., del(5q)). (3) There is an isochromosome of 11q seen from 11q gain and 11p loss, which transitions across the centromere.

4.4.3 | Large CNA calls (>500 kb) and aneuploidies

The copy number filters use a default lower size threshold of 500 kb for calling CNAs with a recommended confidence filter setting of 0.99, resulting in highly confident copy number calls. The copy number size and confidence thresholds can be reduced, but may increase the occurrence of artifactual calls shown by the copy number algorithm. It should be noted that CNAs smaller than 500 kb will still be detected by the SV pipeline, which registers the concomitant structural changes (i.e., deletions, duplications, insertions). Therefore, CNAs identified by the copy number pipeline are large and can be visualized in the copy number track of the Circos Plot (Figure 3B) or in the

Whole Genome Copy Number view. Interstitial copy number changes will almost always be associated with a corresponding SV call (but should be integrated and only reported/counted once) (Figure 3C). Chromosomal aneuploidy (Figure 3B) and whole-arm copy number changes usually do not have an associated SV call (e.g., whole-arm translocations that fuse at the centromeres are currently unresolved due to highly repetitive centromeric sequence and the current lack of reference sequence data in these regions) (Figure 3D).

Occasionally, observable deflections in the copy number track indicating large copy number events or whole chromosome aneuploidies are visible in the Genome View, but may not be shown by the software at recommended settings. These often represent copy number changes at the limit of detection of the technique (Figure 3A) and should be interpreted and reported cautiously, importantly correlating with any relevant clinical data (e.g., sample had very low percentage of abnormal cells, ca. <10%). Changing the CNV or Aneuploidy confidence filter setting to "ALL" will display the relevant information about an observable copy number event that is not called with the "RECOMMENDED" filter setting. Each laboratory should establish a policy for reporting these events.

4.5 | Enumerating "cytogenetically visible" events

Once the results have been filtered and potential artifacts excluded, an assessment of genome complexity can be performed. As done for CMA, OGM-defined complexity can be extrapolated to replicate the complexity defined by CBA. Some considerations for the abnormality count should be considered:

Calls made only by the copy number algorithm: counting abnormalities should be performed as recommended by CMA guidelines,[27,28] considering only those abnormalities larger than 5 Mb.

Intra- and inter-chromosomal rearrangements: Similar to CBA analysis, any cytogenetically visible balanced or unbalanced, intra- or inter-chromosomal rearrangement should be counted. This includes both recurrent disease-specific translocations, but also other rearrangements that would be detected by CBA that are somatic (e.g., Tier 3). Note, that while cryptic translocations would not be counted by CBA (as they are not detected by the technique), they should be counted by OGM.

Catastrophic genome events: These "complex signatures" include well-known catastrophic events globally referred to as chromoanagenesis (chromothripsis, chromoanasynthesis, and chromoplexy)[30,31] as well as those genomic profiles highly enriched in translocations, even if they are not classified in the Tier 1 or Tier 2 categories. The latter likely include marker chromosomes or additional material of unknown origin/significance if detected by CBA, and represent highly rearranged genomes most likely found in a complex karyotype (Figure 3D).

Recurrent clinically significant copy number changes: as described in the standards for interpretation of CNAs and CNA-LOH in neoplasia,[27] sometimes a pattern of copy number changes, rather than an isolated CNA, represents a clinically significant finding (hyperdiploidy, hypodiploidy, or iAMP21). Such patterns should be recognized and often define either specific diagnostic subclasses or prognoses and should be reported as such.

4.6 | Loss of heterozygosity and ploidy analysis

The Bionano Solve RVA does not currently perform LOH analysis and, therefore, cannot be used to directly call changes in ploidy. However, newer software versions will contain this functionality that will be important for detecting ploidy changes and CN-LOH. Use of the de novo assembly can be used to look for regions of homozygosity; however, this analysis requires the sample to have a very high level of abnormal cells. Please see the Section 5.2 in the discussion for more information.

4.7 | Classification of variants using a 4-Tier system

We recommend categorizing genomic variants in neoplastic disorders into four tiers of clinical significance as previously proposed for CNAs in neoplastic samples detected by microarray.[27] However, we recognize that this has not been universally adopted, and, therefore, the decision to use the tiered system is up to the individual laboratory (in accordance with local guidelines or published recommendations). Variants can be evaluated as potential diagnostic, prognostic, or predictive markers. For more detailed description of the 4-Tier system and the criteria for each tier please, see Data S1.

4.8 | Filtering variants with disease specific region files

Region files (lists of clinically relevant chromosomal regions comprised of relevant genes and regions of clinical significance) formulated in BED file format are very useful for rapidly identifying Tier 1 or Tier 2 variants based on disease specific guidelines.[4,5] We recommend assessing large SVs and CNAs (e.g., del(5q), trisomy 8, del(17p), complex genomes) before filtering using the BED file. Once a BED file filter has been selected, the user can select in Access only to display SV calls that overlap regions contained within the BED file. An important consideration is the PRECISION window for SV and CNA calls. While labels are spaced at an average of 6 kb throughout the genome, in some regions, there can be a considerably higher or lower density of labels. Therefore, the selection of the correct amount of "window" on either side of the variant call is critical to make sure your filtering using the BED file does not exclude calls in regions with low label density. We recommend an SV precision setting of 25–50 kb and a CNV precision setting of at least 10 kb. In addition, genes, such as

MYC, are known to have large break point regions (e.g., enhancer hijackers) will require special consideration when designing custom region files. Depending on the lab's specific application, thorough testing of precision settings is recommended on a set of representative samples. It should be noted that the precision setting is intended to capture "displaced" breakpoints in regions of low label density and is not designed to determine the functional consequence of SVs adjacent to relevant genes due to "position-effect." Position effect determination of novel SVs from DNA only based data is, therefore, not recommended.

Region files can be especially helpful for the evaluation of smaller SVs and CNAs. OGM analyses can generate many SV calls that are below the resolution of conventional cytogenetic analysis. In these cases, having a strategy to evaluate these SVs using region files can save both analysis and interpretation time. By using a region file with a list of clinically relevant genes or regions (e.g., disease specific or "pan-cancer" lists), SVs that may change management or have targeted therapies can be identified and included in the report. Conversely, SVs that do not overlap a disease-specific or pan-cancer gene list will likely have limited or no data on which to gauge their clinical significance. As such, these SV calls will be Tier 3 or 4 variants that do not need an interpretation, but may be included in a clinical report at the laboratory's discretion.

Tier 3 and Tier 4 variants may be challenging to distinguish from one another in some situations. However, large intra- or inter-chromosomal rearrangements with VAFs near 0.5 (present in one copy in every cell) that are cytogenetically visible (and that may have reproductive counseling implications) may be reported. Follow up genetic testing (72 h PHA stimulated CBA) is recommended to identify constitutional rearrangements. Smaller submicroscopic SVs, without a convenient orthogonal confirmatory testing, may be reported at the laboratory's discretion. OGM may also be useful in cases where cultured fibroblasts are available for the assessment of germline origin of the SV in question. SVs impacting genes with potential hereditary predisposition to leukemia are currently targets for further investigation.

4.9 | OGM clinical report

Laboratories should establish criteria for which variants they include in the clinical report. The criteria may be different from that used for variant review. For example, laboratories may opt to review all variants that are within the limits of sensitivity of the assay and have sufficient supporting molecule data, but ultimately may choose to only report variants that fall within certain tiers of clinical significance or are above a predetermined size cut-off (i.e., size filtering). Laboratories may also decide to use an established size cut-off for general variant reporting but opt to report smaller variants if they overlap a predetermined list of relevant cancer genes. Another option is to validate OGM as a targeted assay (e.g., as a replacement for a FISH panel) and then only review and report findings that involve the targeted regions (i.e., a region-specific assay). Reporting criteria should be clearly stated in the laboratory protocols and in the report itself. New guidelines for reporting OGM nomenclature have been developed to be included in the updated International Standard for Cytogenetic Nomenclature (ISCN 2024) and as a standalone article.[32] Report sections and sample report layouts can also be found in Table S1.

5 | DISCUSSION

New research in hematologic malignancies regularly uncovers novel clinical relevant cytogenomic biomarkers, many of which cannot be detected by CBA (e.g., cryptic or too small). Cytogenetic and molecular laboratories deal with the growing list of biomarkers by validating separate additional assays/probes. The high sensitivity of OGM to detect structural variants and its high resolution allows for the detection of small and cryptic rearrangements thereby addressing many of the shortcomings of CBA. Importantly, OGM can dynamically adapt to the expanding list of biomarker targets by simply adding the new regions to a gene or region list in an established BED file. This flexibility maintains OGM as a single assay with maximum diagnostic power, which is highly cost effective. These advantages are particularly attractive for small- and medium-size laboratories who often have limited budgetary resources that restrict their ability to continually add new tests.

The performance of OGM in hematological malignancies compared to SOC testing regimens has now been well established with OGM showing equal or better sensitivity and resolution for the detection of diagnostic and prognostic abnormalities malignancies.[8,10–12,33] Consequently, there has been a rapid interest in adopting OGM in clinical laboratories for detection of sentinel cytogenomic abnormalities in patients with hematological neoplasms. As early adopters of the OGM, we recognize that there are still several areas where further developments in technology, in harmonization of reporting criteria and in understanding of both previously undetected SV and the clinical impact of genome complexity need to be better understood. We address some of these issues below.

5.1 | Harmonization of clinical variant reporting

Classification and interpretation of somatic variants according to their impact on clinical care has been widely accepted in clinical practice. This differs from interpretation of germline sequence variations that focuses on the role of a variant in causing a patient's phenotype, for example, "pathogenicity" of a variant for a specific disease. However, classification according to the level of clinical significance is better aligned with the goals of genetic testing in oncology with the goal of identifying "actionable" markers that may be used to optimize the patient's clinical management.

Interpretation of somatic sequence variants in cancer that focuses on their role as diagnostic, prognostic, or predictive (therapeutic) markers has been proposed by multiple professional groups in the USA and Europe. This principle is applied in the joint consensus recommendation between the Association for Molecular Pathology, American College of Medical Genetics and Genomics, American

Society of Clinical Oncology, and College of American Pathologists[26]; in the framework for ranking molecular targets for cancer precision medicine by the European Society for Medical Oncology[34]; and in the classification developed for molecular tumor boards by the German Cancer Consortium (DKTK).[35] Categorization based on the levels of clinical significance has also been proposed for copy number variants and CN-LOH regions in the laboratory standards jointly developed by the American College of Medical Genetics and Genomics and the Cancer Genomics Consortium.[28]

The principles put forward for interpretation of somatic sequence variants, CNAs and CN-LOH, are also applicable for interpretation of structural abnormalities (CNAs and balanced rearrangements) detected by OGM. Variants detected in tumor samples by OGM are potential biomarkers that affect clinical care by impacting diagnosis of a tumor type, prognosis, or selection of a specific treatment (therapeutic/predictive role).

This approach has largely been adopted by American laboratories whilst other countries, for example, in Europe, use the criteria described by Schoumans et al.[28] for the interpretation and reporting of CNA and CN-LOH. These criteria are based on abnormalities predicted to be detectable by CBA (>5 Mb) and small recurrent abnormalities of clinical significance often detected by FISH, MLPA, or CMA. As such the variants are reported in a similar way to CBA reports.

The Consortium also recognizes the need to harmonize interpretation and reporting of cancer variants between published guidelines, variant types, and testing platforms. Aligned recommendations for clinical interpretation, classification, and reporting of different types of cancer variants regardless of their detection method would simplify implementation for clinical laboratories and make reports more understandable to clinicians. It would also foster the use of integrated genomic assays that might simultaneously detect somatic SNVs, indels, CNAs, CN-LOH and balanced chromosomal rearrangements. Reporting results of such assays would be very difficult if disparate sets of rules are used for interpretation of each variant class. OGM presents a unique opportunity to advocate for harmonization in analysis and reporting of SVs due to it being a genome wide assessment with improved resolution and precision compared to conventional cytogenetics.

Based on these considerations, we have detailed a system of interpretation and classification of somatic variants detected by OGM using a framework adapted from the standards and guidelines developed by professional groups for sequence variants, CNAs and CN-LOH in neoplasia.[26–28] These existing classification standards have been developed to be platform neutral; for example, the standards for CNAs and CN-LOH by Mikhail et al.[27] are applicable whether CNAs are detected by CMA, OGM or genome sequencing. We also acknowledge that all laboratories may not wish to report SVs using a tiered system. Nevertheless, the tiered system does provide a relatively consistent way to classify and interpret variants even if it is not used for the final report.

5.2 | Loss of heterozygosity and ploidy

Currently, determination of increased copy number changes at the whole genome level (e.g., triploidy, tetraploidy) and detection of CN-LOH regions are currently limited with the RVA. There are many scenarios where the detection of clinically significant LOH would provide significant additional benefit within an OGM analysis.[36,37] Further improvements to the technology and bioinformatics pipeline are in progress and are anticipated to allow for the identification of CN-LOH below 25 Mb. While not standard protocol, evidence of this utility can be seen by using the de novo assembly which was developed to detect genome wide Absence of Heterozygosity / LOH of ≥25 Mb in germline samples. As an example, in B-cell Acute Lymphoblastic Leukemia, a "hypodiploid-triploid" karyotype (also referred to as "doubled hypodiploid") is a poor prognostic finding where a loss of chromosomes reduces the total chromosome number to a near-haploid or hypodiploid number. This clone then undergoes an endoreduplication resulting in a hyperdiploid or near-triploid number of chromosomes. The original hypodiploid clone can be difficult to detect by CBA but CMA using single nucleotide polymorphisms (SNPs) can readily reveal this diagnostic subcategory of B-ALL by interrogating the characteristic SNP allele patterns which indicate LOH for most chromosomes. For neoplastic samples that have pathognomonic "hypodiploid-triploid" karyotypes, use of the de novo assembly in samples with sufficiently high tumor burden (i.e., >25% VAF) can potentially reveal the characteristic LOH patterns (Figure 4).

5.3 | Genomic complexity and complex karyotypes

CBA is considered the gold standard for identifying complex karyotypes which are a strong predictor of poor prognosis. Complexity interpretation is challenging and criteria defining "complexity" varies between different disease entities.[38–40] Even with CBA, there is no consensus on exactly how chromosome abnormalities should be counted. Further, since complexity is defined at a "microscopic level" it does not account for potentially high levels of complexity that may be seen within a single marker or additional chromosome (by CBA these would be counted as a single abnormality). A higher order of complexity can be detected by CMA which can reveal widespread gains and losses as well as certain genomic signatures, such as chromothripsis, that are considered markers of genomic complexity. Data for risk stratification by CMA is limited and, despite evidence indicating that increasing numbers of CNAs are associated with poorer outcome in entities like CLL, standard criteria for the interpretation of complex CMA data remains outstanding.[41] Schoumans et al.[28] proposed an interpretive model for counting and reporting CNA that could be used in an analogous manner to CBA to calculate complexity. They recommended a cut-off of ≥5 Mb for reporting CNA in addition to any other smaller disease-specific abnormalities. The risk stratification model published by Schoumans et al. has been widely adopted by European laboratories and also validated in a large retrospective study by the European Research Initiative on CLL group.[42] Of note, a recent comparative study by Ramos-Campoy et al.[43] showed that genomic complexity detected by CBA or CMA is not fully equivalent due to the intrinsic limitations of each technique. Further, a similar study comparing CBA and CMA for risk refinement in Myelodysplastic used a similar criterion for copy number assessment and demonstrated changes

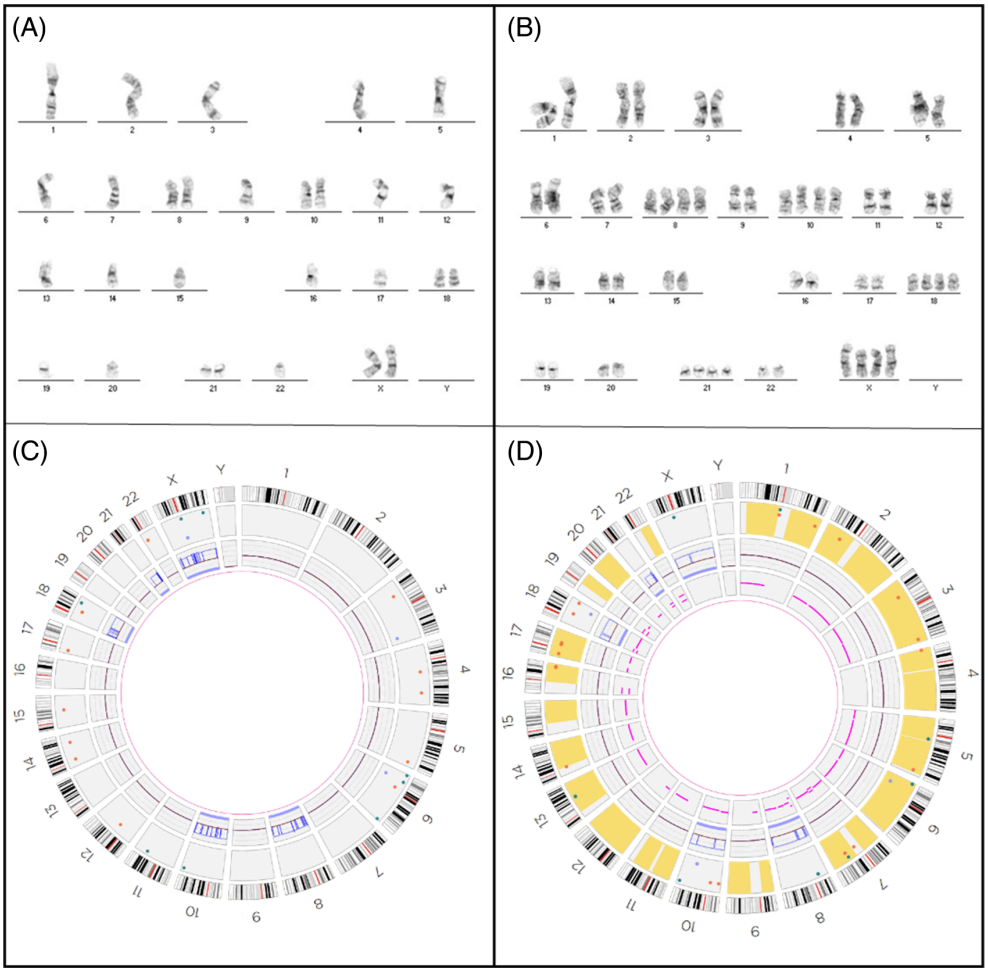

FIGURE 4 Visualization of ploidy changes with the de novo analysis and additional benefit of AOH/loss-of-heterozygosity (LOH) data. A patient with B-cell Acute Lymphoblastic Leukemia (B-ALL). The patient's karyotype was 28,X,+X,+8,+10,+18,+21[10]/56,idemx2[8]/46,XX [3] and both the hyperhaploid abnormal clone and the hyperdiploid abnormal clone are shown in panels A and B, respectively. Patients with B-ALL that suffer a massive chromosomal loss with nearhaploid or low hypodiploid karyotypes often duplicate their hyperhaploid/low hypodiploid clone to a hyperdiploid/triploid clone. These patients, often abbreviated as Ho-Tr, for hypodiploid-triploid have a poor prognosis. Often karyotyping may only reveal the "doubled clone," which can lead to difficulty in accurately assigning prognosis. However, the massive chromosomal loss results in a loss of heterozygosity event for all haploid chromosomes. Optical Genome Mapping results show the chromosomes that remain as two copies in the Rare Variant Assembly (C), however, a de novo assembly with LOH detection clearly shows all of the chromosomes with LOH (yellow regions).

in risk stratification when high resolution techniques are applied.[44] Clearly, our understanding of genomic complexity and its impact in specific disease settings is an area where specific guidance for enumerating complexity and evaluation on patient cohorts within clinical trial settings will be necessary.

To date, only one study has compared the performance of OGM to conventional methodologies in the assessment of genome-wide genomic complexity in patients with Chronic Lymphocytic Leukemia.[18] This study demonstrated that OGM not only effectively detects most of the abnormalities defined by a combination of standard methods (CBA, FISH, and CMA), but also detects a higher number of abnormalities. Furthermore, increasing genome complexity detected by OGM was associated with worse clinical progression.[18] Such findings clearly point to further study of genome complexity by OGM in other hematologic malignancies to better define the "complexity" thresholds and determinants related to patient outcome when using OGM.

6 | CONCLUSIONS

For the most part, diagnostic, prognostic, and predictive biomarkers in hematologic malignancies have been established over many years using CBA. As such, our understanding of how SV impacts these diseases is restricted primarily to abnormalities that are microscopically visible (>10 MB). Recent studies using OGM and NGS clearly indicate that current SOC methodologies do not provide the complete picture of molecular events occurring in our patients. The incomplete profile may explain the variances in outcome often observed, despite the

presence of apparently good prognostic biomarkers. Prospective studies and clinical trials using OGM in patients with a diverse spectrum of hematologic malignancies are needed to not only identify smaller, clinically relevant structural variants, but also to better detect cryptic and previously unidentified cytogenetic abnormalities. OGM is an important tool for discovering novel SVs and disease associations and to build a more complete and thorough understanding of relevant hematologic biomarkers.

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CONFLICT OF INTEREST STATEMENT

NSS and ACS declare a personal financial interest in Bionano Inc. (personal investment). BC is an employee of Bionano Inc. RK declares Honoraria/Travel Funding/Research Support: Illumina, Agena, IBM, QIAGEN, Perkin Elmer, Bionano, PGDx, Cepheid, One Cell Dx, Novartis, AbbVie, and AstraZeneca. All other authors do not declare any conflicts of interest in relation to this manuscript.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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