APPLICATIONS OF NEXT-GENERATION SEQUENCING

Genome structural variation discovery and genotyping

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Abstract | Comparisons of human genomes show that more base pairs are altered as a result of structural variation — including copy number variation — than as a result of point mutations. Here we review advances and challenges in the discovery and genotyping of structural variation. The recent application of massively parallel sequencing methods has complemented microarray-based methods and has led to an exponential increase in the discovery of smaller structural-variation events. Some global discovery biases remain, but the integration of experimental and computational approaches is proving fruitful for accurate characterization of the copy, content and structure of variable regions. We argue that the long-term goal should be routine, cost-effective and high quality *de novo* assembly of human genomes to comprehensively assess all classes of structural variation.

Structural variant
(SV). Genomic rearrangements that affect >50 bp of sequence, including deletions, novel insertions, inversions, mobile-element transpositions, duplications and translocations.

Copy number variant
(CNV). Also defined as unbalanced structural variants; variants that change the number of base pairs in the genome.

Mobile elements
DNA sequences that move location within the genome. Active mobile elements (transposons) in the human genome include *Alu*, L1 and SVA sequences.

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The spectrum of human genetic variation ranges from the single base pair to large chromosomal events, but it has become apparent that human genomes differ more as a consequence of structural variation than of single-base-pair differences[1–6]. Structural variation was originally defined as insertions, deletions and inversions greater than 1 kb in size[7]. With the sequencing of human genomes now becoming routine[8], the operational spectrum of structural variants (SVs) and copy number variants (CNVs) has widened to include much smaller events (for example, those >50 bp in length). The challenge now is to discover the full extent of structural variation and to be able to genotype it routinely in order to understand its effects on human disease, complex traits and evolution.

At least two distinct models have been proposed with respect to associations between disease and structural variation. The first involves large variants (typically gains and losses several hundred kilobase pairs in length) that are individually rare in the population (<1%) but collectively account for a significant fraction of disease, as seen for some neurological and neurocognitive disorders[9–12]. The second includes multicopy gene families that are commonly copy number variable and contribute to disease susceptibility, as seen for traits related to immune gene functions[13,14]. The discovery and genotyping of structural variation has been central to understanding these disease associations. Systematic and comprehensive assessment of structural variation has been problematic owing to the complexity and multifaceted features of SVs. Ideally, SV discovery and genotyping requires accurate prediction of three features: copy, content and structure. In practice, this goal has remained elusive because SVs tend to reside within repetitive DNA, which makes their characterization more difficult. SVs vary widely in size and there are numerous classes of structural variation: deletions, translocations, inversions, mobile elements, tandem duplications and novel insertions (FIG. 1). Within the past 5 years, a variety of computational and experimental methods has emerged; typically each focuses on a particular class of structural variation limited by frequency and size range of the events.

In this Review, we consider current methods for discovery and then for genotyping, including experimental approaches using microarrays, single-molecule analysis and sequencing-based computational approaches. The distinction between discovery and genotyping is important. Once a variant has been detected, validated and characterized at the sequence level (discovery), a different suite of methods may be applied to infer genotypes with relaxed thresholds. We discuss recent advances in the genetic characterization of germline structural variation — recognizing that the methods may be applied, in principle, to the study of somatic structural variation — and highlight current deficiencies, as well as areas for future development.

Hybridization-based microarray approaches

Microarrays have been the experimental workhorse of CNV discovery and genotyping[1,6,15–18]. These are represented primarily by array comparative genomic hybridization (array CGH) and SNP microarrays. Both hybridization-based

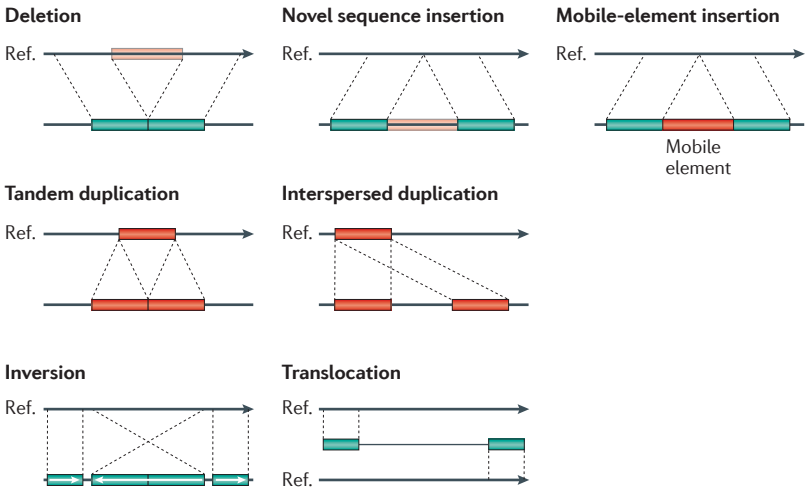


Figure 1 | **Classes of structural variation.** Traditionally, structural variation refers to genomic alterations that are larger than 1 kb in length, but advances in discovery techniques have led to the detection of smaller events. Currently, >50 bp is used as an operational demarcation between indels and copy number variants (CNVs). The schematic depicts deletions, novel sequence insertions, mobile-element insertions, tandem and interspersed segmental duplications, inversions and translocations in a test genome (lower line) when compared with the reference genome.

technologies infer copy number gains or losses compared to a reference sample or population, but differ in the details and application of the molecular assays.

Array CGH. Array CGH platforms are based on the principle of comparative hybridization of two labelled samples (test and reference) to a set of hybridization targets (typically long oligonucleotides or, historically, bacterial artificial chromosome (BAC) clones). The signal ratio is then used as a proxy for copy number (see BOX 1 for details). An important consideration is the effect of the reference sample on the copy-number profile. For example, when only one sample is examined, a loss in the reference sample is indistinguishable from a gain in the test sample. For this reason, a well-characterized reference is key to interpretation of array CGH data[19]. Early studies of germline CNVs were based on BAC arrays or low-resolution oligonucleotide platforms and allowed detection of CNVs typically greater than 100 kb[1,2,6] (BOX 2). These initial studies highlighted the incredible number of CNVs observed in healthy individuals; however, the breakpoints of these alterations were not sufficiently well-defined to allow accurate assessment of the proportion of the genome altered or its gene content. This led to a drastic overestimation of the extent of copy-number polymorphism using large-insert BAC clones[2], which was subsequently refined by oligonucleotide microarrays or sequence-based studies of the same DNA samples[4,5,20,21].

Currently, Roche NimbleGen and Agilent Technologies are the major suppliers of whole-genome array CGH platforms and routinely produce arrays with up to 2.1 million (2.1M) and 1M long oligonucleotides (50–75-mers), respectively, per microarray. Detection of a CNV typically requires a signal from at least 3 to 10 consecutive probes (BOX 1); as a result, SNP and CGH microarrays can routinely detect anywhere from dozens to several hundred events per genome depending on the platform applied (BOXES 1,2). Two studies have recently used ultra-high-resolution arrays (24M to 42M probes) for array CGH-based SV discovery in samples from HapMap individuals[5,19]. Although such high-density arrays are not practical for a large number of samples (30 and 40 samples were used in these studies), these approaches enabled the discovery of CNVs down to 500 bp, with breakpoints precise enough to allow the identification of sequence motifs at a subset of variants. One key advantage of array CGH platforms is the availability of custom, high-probe-density arrays from both major manufacturers. This has led to their widespread adoption in clinical diagnostics, essentially replacing karyotype analysis as the primary means of detecting copy-number alterations among children with developmental delay[22].

SNP arrays. SNP microarray platforms are also based on hybridization, with a few key differences from CGH technologies. First, hybridization is performed on a single sample per microarray, and log-transformed ratios are generated by clustering the intensities measured at each probe across many samples[20,23,24]. Second, SNP platforms take advantage of probe designs that are specific to single-nucleotide differences between DNA sequences, either by single-base-extension methods (Illumina) or differential hybridization (Affymetrix)[20,23,24]. One key disadvantage is that, per probe, SNP microarrays tend to offer lower signal-to-noise ratio than do the best array CGH platforms. This is apparent in comparisons of array CGH and SNP platforms in terms of detection of CNVs by a purely ratio-based approach[24–27]. However, a key advantage of SNP microarrays is the use of SNP allele-specific probes to increase CNV sensitivity, distinguish alleles and identify regions of uniparental disomy through the calculation of a metric termed B allele frequency (BAF) (BOX 1).

SNP arrays have proved popular in CNV-detection studies, historically as complements to array CGH platforms for fine-mapping regions[2] and currently in the large-scale discovery of CNVs in a broad variety of populations[16,20,23,28,29]. Early SNP arrays demonstrated poor coverage of CNV regions, but recent arrays (such as the Affymetrix 6.0 SNP and Illumina 1M platforms) incorporate better SNP selection criteria for complex regions of the genome and non-polymorphic copy-number probes (which are examined for log ratios but not BAF)[20,23,30]. Another important consideration is the choice of population because the average heterozygosity affects the proportion of SNPs that will generate a meaningful BAF signal (typically, heterozygosity is 30–40% in Illumina platforms). This is particularly relevant when dealing with populations that may have experienced a drastic bottleneck, as opposed to more outbred populations, and thus may affect the number of probes needed to identify an alteration[23,24]. Some studies combine array CGH and SNP platforms to offer higher confidence in CNV detection[2,20,30].

Array comparative genomic hybridization
(Array CGH). A technique based on competitively hybridizing fluorescently labelled test and reference samples to a known target DNA sequence immobilized on a solid glass substrate and then interrogating the hybridization ratio.

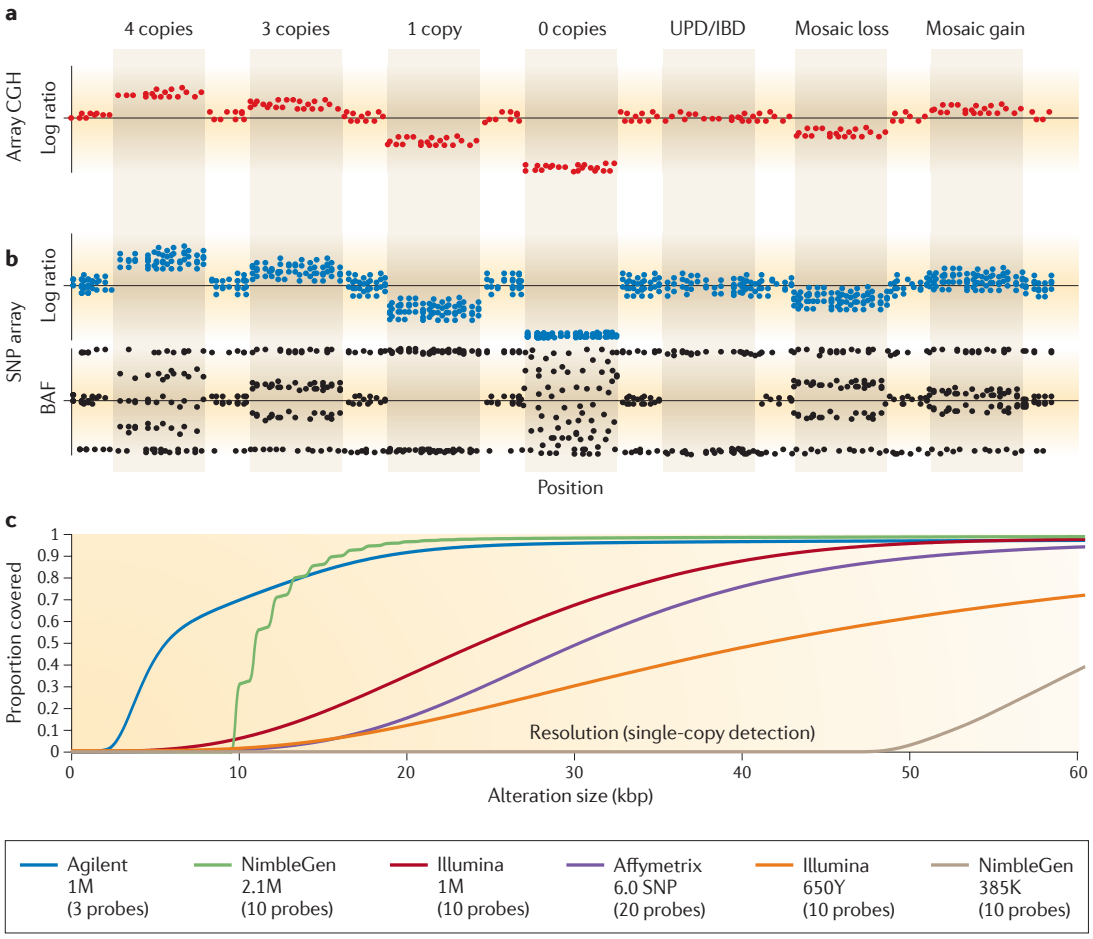
SNP microarrays
Hybridization-based assays in which the target DNA sequences are discriminated on the basis of a single base difference. Assays are processed with a single sample per array and perform both SNP genotyping and copy-number interrogation.

Single-base extension
Single-base-extension reactions use a primer that binds to a region of interest and follow this with an extension reaction that allows the incorporation of a single base after the primer.

Box 1 | **Array CGH versus SNP microarray detection**

In array comparative genomic hybridization (array CGH), the signal ratio between a test and reference sample is normalized and converted to a $\log_2$ ratio, which acts as a proxy for copy number[18,25,112]. An increased $\log_2$ ratio represents a gain in copy number in the test compared with the reference; conversely, a decrease indicates a loss in copy number (see the figure, part **a**). SNP arrays generate a similar metric by comparing the signal intensities for the sample being analysed to a collection of reference hybridizations, or the rest of the population being analysed (part **b**, upper panel). The log ratio metric for SNP arrays demonstrates a lower per-probe signal-to-noise ratio (SNR) than array CGH (compare **a** and **b** in the figure); however, SNP arrays offer an additional metric that enables a more comprehensive assignment of copy number than does array CGH. This metric, termed B allele frequency (BAF) (part **b**, lower panel), can be calculated as the proportion of the total allele signal (A + B) explained by a single allele (A). The BAF has a significantly higher per-probe SNR than the log ratio data and can be interpreted as follows: a BAF of 0 represents the genotype (A/A or A/–), whereas 0.5 represents (A/B) and 1 represents (B/B or B/–). Different BAF values occur for AAB and ABB genotypes or more complex genotypes (for example, AAAB, AABB and BBBA). Homozygous deletions result in a failure of the BAF to cluster[23,24]. Thus, the BAF may be used to accurately assign copy numbers from 0 to 4 in diploid regions of the genome. The BAF also allows detection of copy-neutral events such as segmental uniparental disomy (segmental UPD) or whole-chromosome UPD and identity by descent (IBD), which results when a segment of one chromosome is replaced by the other allele without a change in copy number (this is therefore not detectable by array CGH)[24]. An additional advantage of the BAF is that it can be used to reliably detect and type low-level mosaic gains and losses[24,113,114] (see the figure, part **b**).

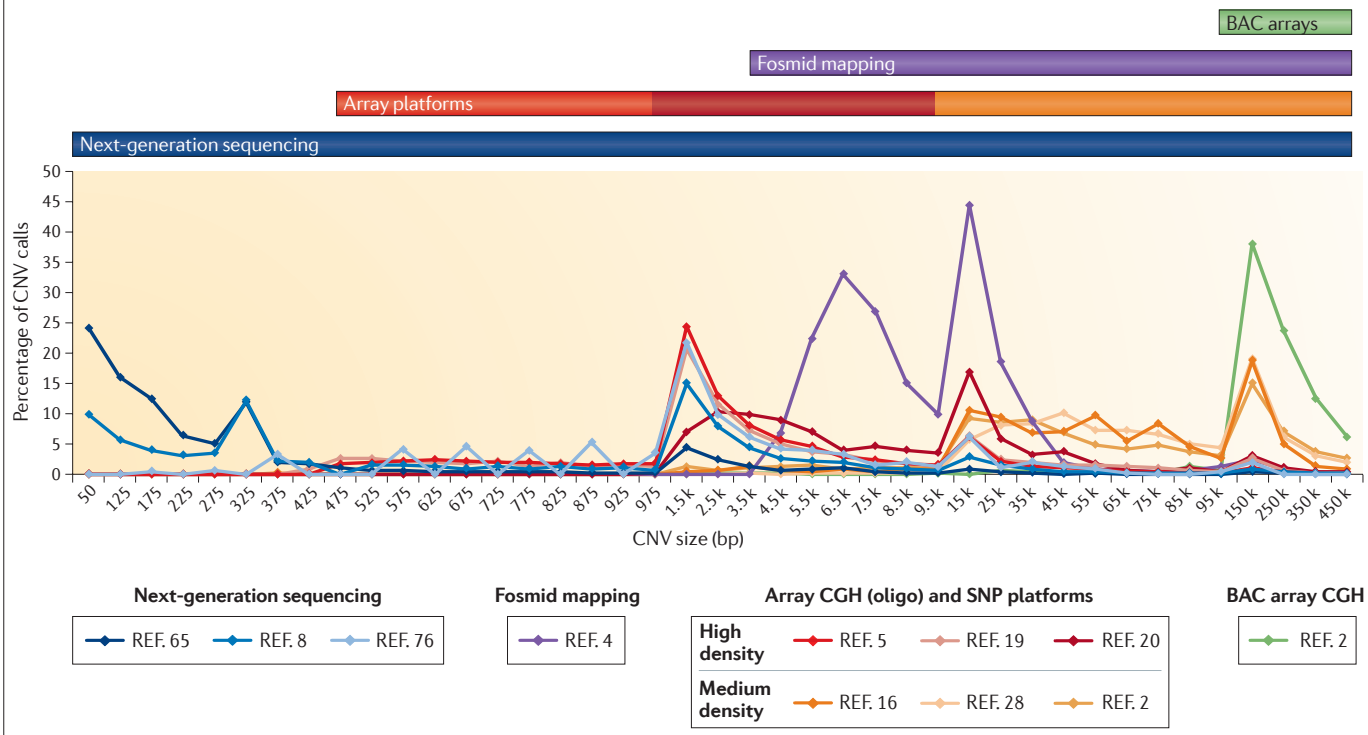
Another important consideration in choosing an array platform is the ability to detect alterations in the size range being investigated. Array resolution is complicated by non-uniform probe distributions and differing SNRs between platforms, and as a result two platforms cannot be compared by simply counting the number of probes included. The number of probes required to detect a single-copy alteration varies between platforms, with Agilent Technologies offering the highest per-probe performance[25,26,32]. Part **c** of the figure shows the probe coverage of several major array platforms as determined by ResCalc[25]. This represents the theoretical ability to detect a copy number variant at any given location in the genome. In practice, however, thresholds of copy-number detection are typically greater owing to variable probe performance (BOX 2). Although alterations can, theoretically, be detected with a single probe using the Agilent platform, we set the detection limit to a more realistic (in a discovery context) three probes. The other major array platforms tend to require more probes, with Roche NimbleGen[34] and Illumina[16] platforms requiring ten probes, and Affymetrix[39] requiring 20 probes to reliably detect a single-copy alteration.



Segmental uniparental disomy
Uniparental disomy (often abbreviated UPD) is a cryptic alteration in which two copies of a chromosome or segment (segmental UPD) are present, but derive from a single parent.

Limitations. Microarrays are limited to detecting copy-number differences of sequences present in the reference assembly used to design the probes[31], provide no information on the location of duplicated copies and are generally unable to resolve breakpoints at the single-base-pair level. Both array CGH and SNP platforms tend to suffer reduced sensitivity in the detection of single-copy gains (3 to 2 copy-number ratio) compared with deletions (1 to 2 copy-number ratio)[16,23,25,32]. This is particularly challenging when gains encompass only a few probes and SNP arrays may not contain sufficient probe density to use the BAF measurement. Thus, smaller

Box 2 | **Copy number variant size distribution and discovery methods**



Different copy number variant (CNV) discovery methods are better able to identify CNVs of different sizes. The figure shows the proportion of CNV calls within a given size range for several recently published discovery efforts. Each of the different platforms tends to detect the largest proportion of events at the smallest size ranges to which they are sensitive, as expected owing to the increasing frequency of CNVs at smaller sizes. In terms of performance, next-generation sequencing (NGS)[8,65,76] offers the widest possible range of detection, followed by ultra-high-resolution arrays (42 million (42M) probe, Roche NimbleGen[5], and 24M probe, Agilent Technologies[19]), high-resolution SNP arrays[20] and fosmid end-sequence pair mapping[4], and finally medium-density oligonucleotide microarrays[2,16,28] and bacterial artificial chromosome (BAC) platforms[2]. The size ranges detectable by each platform are illustrated by coloured bars above the plot indicating the lower limit of detection. Local peaks at 1 kb, 15 kb and 150 kb are due to changing bin sizes in the plot.

In addition to the size range of alterations detected, it is important to consider the differential abilities of assays to detect multiple subtypes of genomic alterations. The table shows the numbers of events detected for various categories of genomic alterations — deletions, novel insertions, inversions, duplications and mobile-element insertions (MEIs) — as reported in the database dbVar for the associated publications[42]. This highlights the significant bias of array platforms to deletion events, as well as the use of fosmid and sequencing-based platforms in detecting events missed by array technologies. Array CGH, array comparative genomic hybridization; CNP, copy-number polymorphism; ESP, end-sequence pair.

Method	Samples	Deletions		Novel insertions		Inversions		Duplications		MEIs		Refs
		Calls	Median length	Calls	Median length	Calls	Median length	Calls	Median length	Calls	Median length	
SNP microarray*	270	1,122	6,216	-	-	-	-	442	14,122	-	-	20
SNP microarray‡	2,493	9,963	50,265	-	-	-	-	3,880	108,336	-	-	16
Fosmid ESP	8	1,843	8,657	560	7,594	1,146	77,119	1,768	8,429	-	-	4
Array CGH§	40	7,909	2,284	-	-	-	-	4,740	5,265	-	-	5
Array CGH‖	30	14,597	2,439	-	-	-	-	5,502	3,835	-	-	19
NGS	185	22,025	742	128	98	-	-	501¶	138	5,371	291	8

*Affymetrix 6.0 SNP (CNP calls only). ‡Illumina 300K, 550K and 650K. §Custom 42M probe, NimbleGen (unique CNV loci). ‖Custom 24M probe, Agilent. ¶Tandem duplications only.

events detected by array platforms are overwhelmingly deletions, partly owing to an ascertainment bias[5,16,20]. Homozygous deletions are the easiest class to detect regardless of platform and can be detected with fewer probes than single-copy gains and losses[23,25].

An important practical consideration is that the various commercial array platforms offer different probe densities and per-probe performance in detecting alterations[25,26,32–35] (BOX 1). Not surprisingly, numerous copy-number detection algorithms are available to call CNVs from microarrays, which, depending on parameter optimization, can lead to significant differences in detection and complications in downstream interpretation[30,36–41]. Current recommended solutions include using consensus calls from multiple algorithms[30], using multiple samples to refine CNV calls[20,39], selecting algorithms designed specifically for the platform being examined[30,41] and optimizing parameters in conjunction with manual curation for a subset of events[16,41]. Although most algorithms and microarray platforms perform comparably for the detection of large events, smaller events are more challenging to routinely detect, with all single microarray array platforms (except custom arrays targeted to specific loci) losing sensitivity below 10 kb (BOX 2). As a result, small events are under-represented in databases such as dbVar[42] (BOX 2) and the systematic discovery of pathogenic CNVs below 25 kb remains unexplored in most studies of disease[16,43,44].

Perhaps the most important limitation of arrays is the use of hybridization-based assays in repeat-rich and duplicated regions. Array CGH and SNP platforms assume each location to be diploid in the reference genome, which is not valid in duplicated sequence. The signal for a 5 to 4 copy ratio, or other complex patterns, will not fit the expected results for a diploid reference sequence and may drop below the assay's sensitivity to discriminate signals[15]. This is particularly challenging because CNVs have a strong positive correlation with segmental duplications and many breakpoints lie in duplicated regions[5,15,16,45]. Consequently, the accurate boundaries and copy numbers of these events will require additional technologies.

Advantages. Microarrays offer a distinct advantage in terms of throughput and cost. Large CNVs are individually very rare in the general population, yet 8% of individuals have a CNV of >500 kb in their genome[16]. Determining the pathogenic significance of any particular event in a rare-variant disease model requires screening of thousands of affected individuals and controls. Given the low cost of array CGH and SNP platforms and the large collection of public SNP data available from genome-wide association studies[46], microarray data provide an opportunity to assay the CNV landscape of large data sets. For example, an analysis of 2,493 Illumina SNP profiles was used to generate a comprehensive picture of large CNVs in the 0.5–1% frequency range[16]. Expansion of sample sizes in future studies will aid the design of genotyping assays to examine even larger populations and increase our understanding of human disease.

Single-molecule analysis

Microarray approaches cannot identify balanced structural variants or, in the case of duplication, specify the location of a duplicated sequence (BOX 2); understanding the structure and location has traditionally required visualization at the single-molecule level. Approaches such as fluorescent *in situ* hybridization (FISH), fiber-FISH and spectral karyotyping provided our first glimpses of common and rare genome structural variation[47]. However, their low throughput and low resolution limit their application to a few individuals and to particularly large structural differences (~500 kb to 5 Mb). To improve resolution and scalability, many groups are developing methods for the direct visualization of structure in stretched DNA fragments at a large scale. For example, optical mapping, a technique originally developed to analyse yeast genomes[48], was recently applied to human genome SV analysis[49]. This method is based on a modification of traditional restriction mapping, wherein restriction digestion is performed on immobilized DNA; this allows the identification of the fragment sizes and changes in their relative order on the basis of comparison to an *in silico* digested version of the reference genome sequence. This powerful technique allows fine-scale structural analysis of genomes, detecting inversions and translocations, as well as copy-number alterations, and their locations[4,49,50]. Although capable of detecting novel insertions, the technique is limited by its dependence on a reference genome, and currently has very limited throughput.

DNA barcoding methodologies are a promising alternative that may one day allow high-throughput detection of balanced structural differences; these methods include scanning fluorescently nick-labelled DNA molecules in nano-channel flow cells[51] or nanoslits[52] and the use of SNP-specific labelling of stretched DNA for haplotype resolution[53]. Similarly, absolute copy-number estimations made by amplifying single molecules using emulsion picolitre droplet PCR[54] or single-molecule sequencing of human genomes[55] offer tremendous potential to understand structural changes at the cellular level.

Sequencing-based computational approaches

The advent of next-generation sequencing (NGS) technologies[56–59] promises to revolutionize structural variation studies and, ultimately, replace microarrays as the platform for discovery and genotyping. However, NGS approaches present substantial computational and bioinformatics challenges. Most of the current algorithms for SV discovery are modelled on computational methods that were first developed to analyse capillary sequencing reads and fully sequenced large-insert clones[3,60]. There are four general types of strategy[61,62], all of which focus on mapping sequence reads to the reference genome and subsequently identifying discordant signatures or patterns that are diagnostic of different classes of SV (described below and shown in FIG. 2).

Read-pair technologies. Read-pair methods assess the span and orientation of paired-end reads and cluster 'discordant' pairs in which the mapping span and/or

Nano-channel flow cells
Specialized flow cells narrow enough for a single DNA molecule to pass through in linear form without having sufficient room to fold over on itself.

Nanoslits
Narrow channels (~1 μm wide) on specialized silicon substrates. They are loaded with linear stretched DNA strands by applying a charge to microchannels on the substrates that contain electrodes.

Emulsion picolitre droplet PCR
Emulsion PCR is based on the generation of independent PCR reaction by emulsifying the aqueous reagents in oil such that each droplet becomes a separate PCR reaction. Reagents are diluted such that each droplet contains a single target sequence.

Paired-end reads
Two reads sequenced from the start and end of the same molecule (such as a fosmid, bacterial artificial chromosome or next-generation sequence fragment).

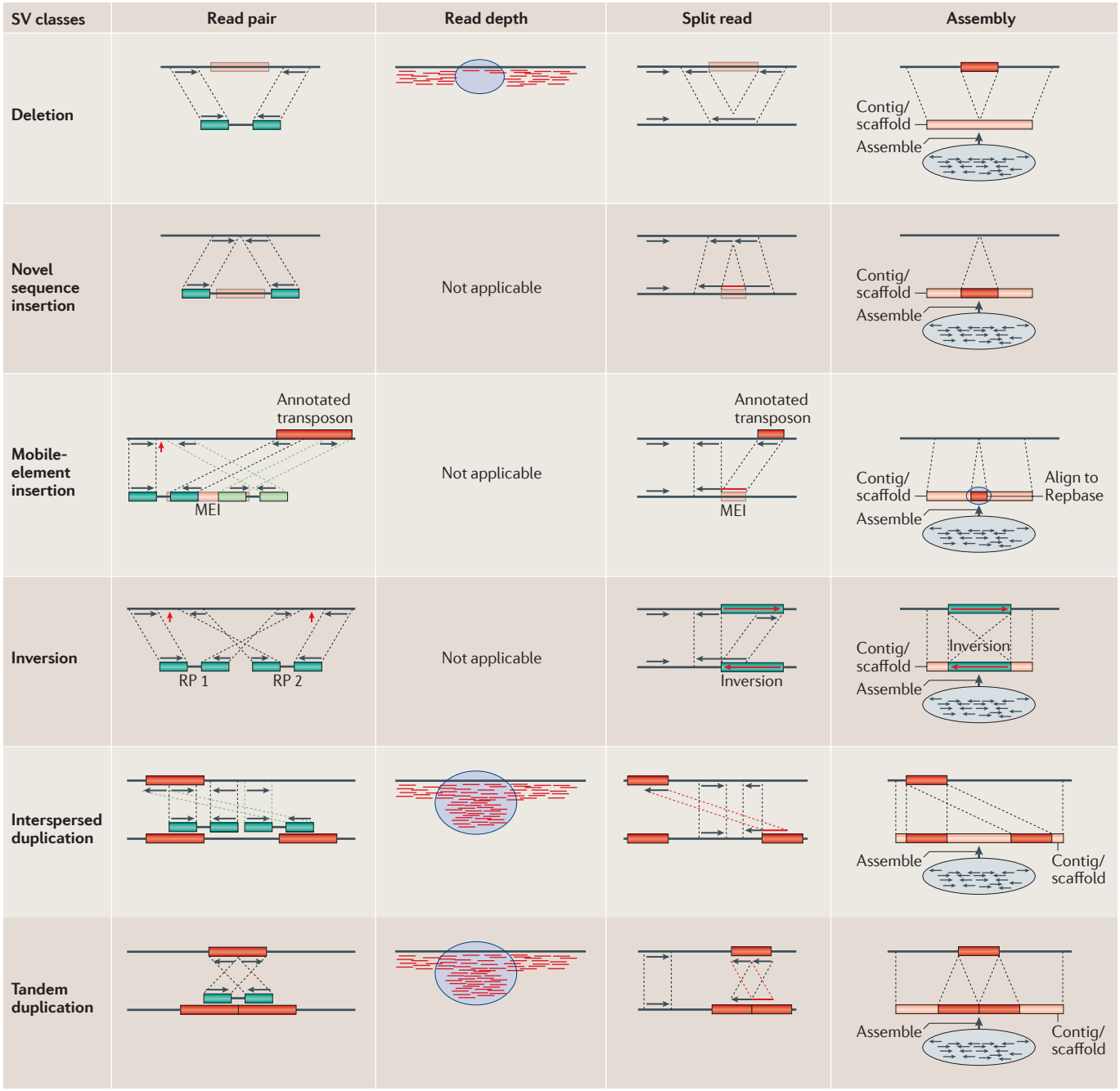


Figure 2 | **Structural variation sequence signatures.** There are four general sequence-based analytical approaches used to detect structural variation. Theoretically, read-pair (RP), split-read and assembly methods can be used to discover variants from all classes of structural variant (SV), but each has different biases depending on the underlying sequence content of the variants and the data properties of the sequence reads. However, read-depth approaches can be used to detect only losses (deletions) and gains (duplications), and cannot discriminate between tandem and interspersed duplications. Briefly, read-pair methods analyse the mapping information of paired-end reads and their discordancy from the expected span size and mapped strand properties. Sensitivity, specificity and breakpoint accuracy are dependent on the read length, insert size and physical coverage[3,4,59,62,65,66,68,69]. Breakpoints are indicated by red arrows. Read-depth analysis examines the increase and decrease in sequence coverage to detect duplications and deletions, respectively, and predict absolute copy numbers of genomic intervals[45,62,74–76]. Split-read algorithms are capable of detecting exact breakpoints of all variant classes by analysing the sequence alignment of the reads and the reference genome; however, they usually require longer reads than the other methods and have less power in repeat- and duplication-rich loci[62,78,79]. Assembly algorithms[83–86,115] have the most power to detect SVs of all classes at the breakpoint resolution, but assembling short sequences and inserts often result in contig/scaffold fragmentation in regions with high repeat and duplication content[89]. MEI, mobile-element insertion. Repbase is a database of repetitive elements.

orientation of the read pairs are inconsistent with the reference genome (FIG. 2). Most classes of variation can, in principle, be detected. Read pairs that map too far apart define deletions, those found too close together are indicative of insertions, and orientation inconsistencies can delineate inversions and a specific class of tandem duplications[3,4,59,63]. Read pairs in which only one end clusters and the others do not map to the reference have been used to flag variant sequences not included in the reference genome (novel insertions). The read-pair method is the most widely applied approach and was first demonstrated using BAC end sequences generated from the breast cancer cell line MCF-7 (REF. 60). It was subsequently applied to germline genetic variation using a fosmid end sequence library[3]. Later, it was applied to next-generation, paired-end data generated by the 454 FLX platform[59]. There are now many computational tools based on a read-pair approach, including PEMer[64], VariationHunter[65–67], BreakDancer[68], MoDIL[69], MoGUL[70], HYDRA[71], Corona[58] and SPANNER (REFS 8,62 and C. Stewart and colleagues, personal communication).

Read-depth methods. Read-depth approaches assume a random (typically Poisson or modified Poisson) distribution in mapping depth and investigate the divergence from this distribution to discover duplications and deletions in the sequenced sample[45]. The basic idea is that duplicated regions will show significantly higher read depth and deletions will show reduced read depth when compared to diploid regions (FIG. 2). Read-depth approaches using NGS data were first applied to define rearrangements in cancer[72,73], and segmental duplication and absolute copy-number maps in human genomes[74,75]. Methods that attempt to discover smaller deletions and duplications at better breakpoint resolution include the event-wise-testing (EWT) algorithm[76] and CNVnator[8,62,77].

Split-read approaches. Split-read methods are capable of detecting deletions and small insertions down to single-base-pair resolution and were first applied to longer Sanger sequencing reads[78]. The aim is to define the breakpoint of a structural variant on the basis of a 'split' sequence-read signature (that is, the alignment to the genome is broken; a continuous stretch of gaps in the read indicates a deletion or in the reference indicates an insertion; FIG. 2). Extensions of this approach may also detect mobile-element insertions (MEIs) if the reads are sufficiently long to span the mobile element (for example, >400 bp for *Alu* elements)[62] to characterize the full sequence content. Alternatively, if the read length is shorter but the MEI breakpoint is in a unique sequence, a split-read approach can be used to anchor the insertion[62]. Application of this method to NGS data sets is currently limited owing to the difficulty in aligning shorter reads; however, the Pindel algorithm[79] uses paired-end reads to reduce the search space for potential split reads, thus reducing the computational overhead of the local gapped alignment of short sequences to the reference genome.

Fosmid end sequence library
Paired-end sequences from a collection of bacterial cloning vectors that can carry an average of 40 kb of DNA.

Sequence assembly. In theory, all forms of structural variation could be accurately typed for copy, content and structure if the underlying sequence reads were long and accurate enough to allow *de novo* assembly. In practice, sequence-assembly approaches are still in their infancy and typically use a combination of *de novo* and local-assembly algorithms to generate sequence contigs that are then compared to a reference genome (FIG. 2). Local sequence assembly of fosmid clones with discordant read pairs has been used to systematically discover structural variation in 17 human genomes[4,31,63] (BOX 2). Approaches that involve library construction, clone array and end sequencing are too laborious and prohibitively expensive to be widely adopted. Ideally, complete genome sequencing followed by *de novo* assembly and comparison to a high-quality reference could identify thousands of structural variants. For example, a genome assembly from capillary sequence reads from a human individual has been used to characterize 12,178 structural variants[80–82]. Well-known *de novo* assembly algorithms for next-generation whole-genome shotgun (NG-WGS) data include EULER-USR[83], ABySS[84], SOAPdenovo[85] and ALLPATHS-LG[86]. Using the Cortex assembler[8,62], variant assembly can be done entirely *de novo* or with different degrees of information from a reference, and Cortex has the ability to simultaneously assemble multiple genomes and call SVs between samples without the need for a reference. The NovelSeq framework[87] merges *de novo* and local-assembly methods to characterize novel sequence insertions and, finally, TIGRA[8,62] aims to improve breakpoint estimations in SV discovery.

Limitations. None of the four main approaches to discovering structural variation using sequence data is comprehensive. When many algorithms and experimental methods are applied to the same DNA samples, a significant fraction of the validated variants remains unique to a particular approach (FIG. 3). Each method has different strengths and weaknesses in detection, depending on the variant type or the properties of the underlying sequence at the SV locus. Although read depth is the only sequencing-based method to accurately predict absolute copy numbers[74,75], the breakpoint resolution is often poor. Read-pair approaches are powerful, but resolving ambiguous mapping assignments in repetitive regions is challenging and accurate prediction of SV breakpoints depends on very tight fragment size distributions, which can make library construction difficult and costly[61]. On the basis of typical NGS fragment sizes, more than 90% of the discovered events are less than 1 kb and most of these are deletions rather than insertions[8,62] (BOX 2). Similarly, split-read algorithms can be devised to detect a wide range of SV classes with exact breakpoint resolution; however, split read is currently reliable only in the unique regions of the genome. Sequence assembly promises to be the most versatile method by facilitating pair-wise genome comparisons; however, it has been shown to be heavily biased against repeats and duplications owing to assembly collapse over such regions[88,89]. Its application to SV detection is not routine and will require substantial development. The realization that the

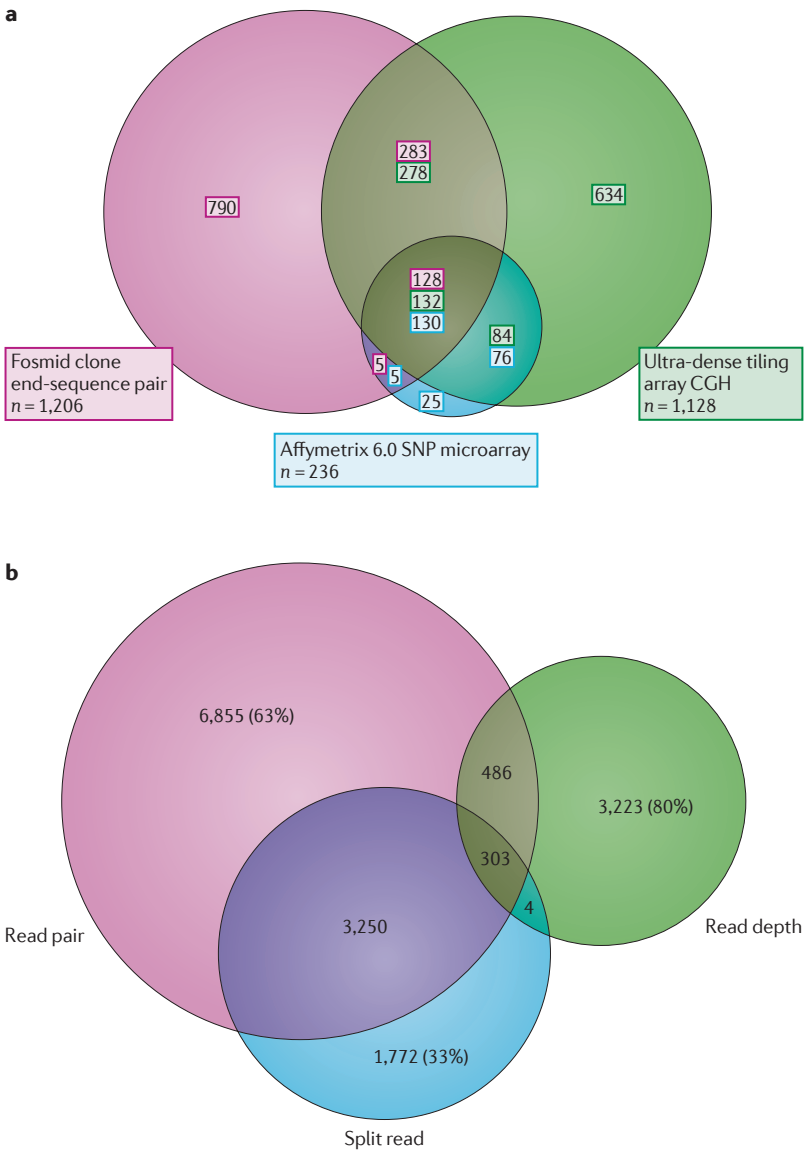


Figure 3 | **Copy number variant discovery biases. a** | Three different technologies have been applied to copy number variant (CNV) discovery for DNA obtained from the same five individual genomes (NA18517, NA19240, NA12878, NA19129 and NA12156). The experimental methods are: fosmid paired-end sequencing[4,63], array comparative genomic hybridization (array CGH)[5] and SNP microarray genotyping[20]. In this Venn diagram, only copy-number gains and losses of >5 kb are compared. SNP microarray CNVs in this study[20] are biased towards common copy-number polymorphisms, which explains, in part, the fewer calls and the greater overlap with the other data sets. The fosmid end-sequence pair method also detects inversions, which are not considered in this analysis. **b** | This Venn diagram shows the numbers of unique and shared structural variants (SVs) found by different sequencing-based discovery approaches that have been used in the 1000 Genomes Project and shows that the approaches are complementary[62]. Read-pair, read-depth and split-read methods (involving 14 distinct algorithms) were applied to the same 185 genomic DNA samples. The proportion of the total number of SVs discovered by one approach that is unique to that approach may be as high as ~80%. Read-pair and split-read methods show the greatest extent of overlap. Read depth and split read are the most discordant approaches, with fewer than 20% of SVs detected by one approach detected by the other (assembly approaches are not compared as they are still in the development stage). The main differences in SV detection between these approaches are primarily found in duplication- and repeat-rich regions. Part **a** is modified, with permission, from REF. 63 © (2010) Elsevier. Part **b** is modified, with permission, from REF. 62 © (2011) Macmillan Publishing Ltd. All rights reserved.

computational approaches outlined above can discover only a subset of structural variants and have various biases in detection has prompted the recent development of algorithms that incorporate multiple methodologies to improve sensitivity and specificity. Three algorithms, SPANNER[8,62], CNVer[90] and Genome STRiP[91], combine read-pair and read-depth methods in different ways to more reliably detect CNVs.

Perhaps the greatest problem in using NGS to discover structural variation is the nature of the data. Sequence reads generated by the NGS platforms are considerably shorter than those produced by the capillary-based methods. Owing to the complex nature of human genomes (for example, widespread common repeats and segmental duplications), there is considerable read-mapping ambiguity. Longer reads and inserts are needed to ameliorate this bias by increasing the specificity in read mapping. It is estimated, however, that >1.5% of the human genome cannot be covered uniquely even with read lengths of 1 kb[92]. Another concern is sequence coverage, defined as the average number of times each base pair in the genome is represented in an aligned read. Sequence coverage is an important factor in achieving high sensitivity and specificity in SV detection (see below). Some projects may opt to sequence samples at low coverage for cost efficiency (for example, the 1000 Genomes Project uses two- to sixfold coverage[8]); however, this reduces the power to discover structural variation. To help ameliorate this effect, the read-pair-based MoGUL algorithm[70] pools the mapping data of several individuals to detect common CNVs in a population (this will, however, still have reduced sensitivity to lower-frequency variants). A newer version of VariationHunter[67] uses a similar pooling strategy, yet provides more sensitivity in detecting rare variation.

Finally, storage and analysis of NGS data requires a substantial investment in computational resources. Today, the raw sequencing data are stored in FASTQ files that contain a minimal amount of information: read name, sequence and the associated quality values. There is an urgent need for improvements in the efficiency of data processing as it is projected that the number of sequenced genomes generated worldwide will exceed 30,000 by the end of 2011 (REF. 93). Public access to such data would provide a rich resource for SV discovery.

Advantages. The most important benefit of NGS technologies is that it is possible to discover a multitude of variant classes (BOX 2) with a single sequencing experiment. In addition, the sequence data are largely unbiased and present a potential for understanding the complete spectrum of genetic variation. Genome-wide analysis without *a priori* information is possible, and the specificity and linear dynamic range response of NGS data offer many advantages for estimation of copy number. Through analysis of read depth, and uniquely identifying paralogous sequence variants (termed singly unique nucleotides (SUNs)), we can now begin to accurately estimate the absolute copy number of duplicated regions of the human genome[74,75] (FIG. 4). Characterization of absolute copy numbers and the ability to distinguish

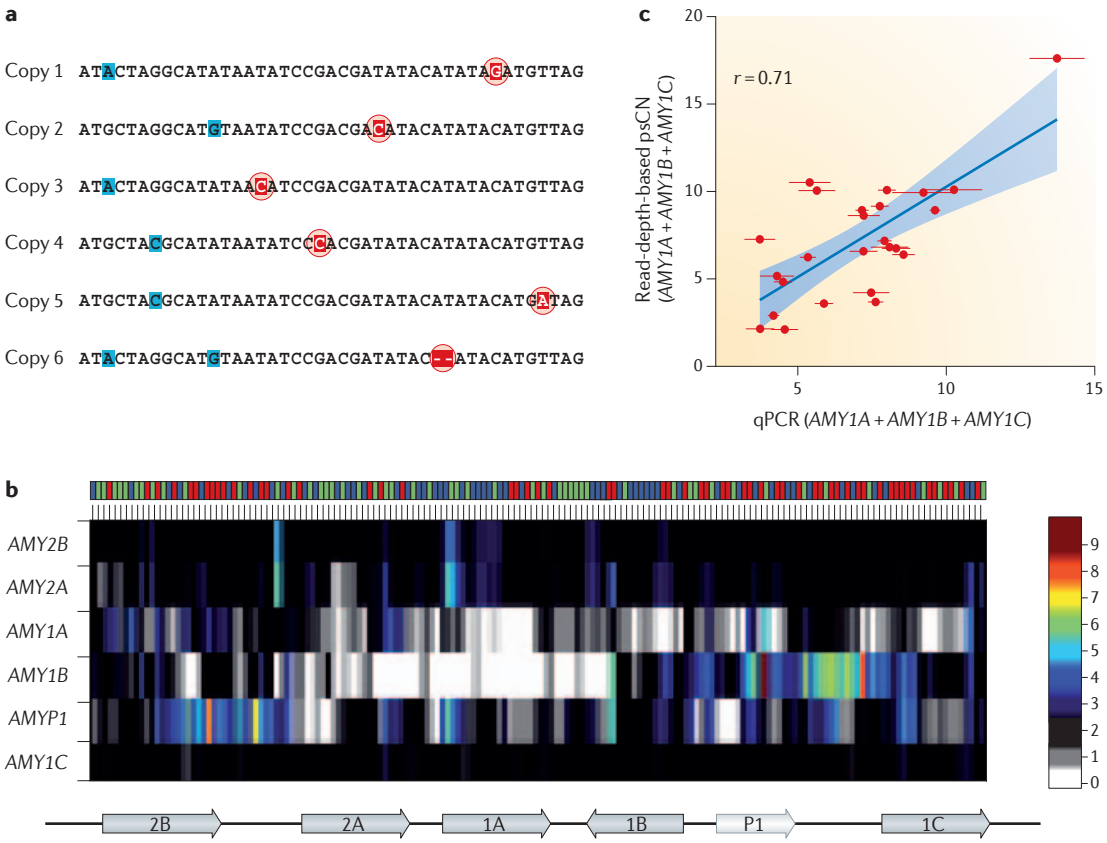


Figure 4 | **Genotyping duplicated paralogues using next-generation sequencing. a** | Singly unique nucleotide (SUN) identifiers that distinguish paralogues from each other (red) are shown in the multiple sequence alignment of duplicated genes. These are distinguished from paralogous sequence variants that are not unique to a specific copy (blue). **b** | Read depth is measured at the SUN positions and used to estimate the copy number of each specific member of the amylase gene family. Across the top, each column represents a different individual from the 1000 Genomes Pilot Project. The colours represent the population identifiers: YRI (Yoruba in Ibadan, Nigeria) is shown in blue; CEU (Utah residents with northern and western European ancestry) is shown in green; and CHB/JPT (Chinese from Beijing, China, and Japanese from Tokyo, Japan) is shown in red. The corresponding copy-number prediction is depicted as a heat map. The pancreatic amylase genes (*AMY2A* and *AMY2B*) show little variation compared with the salivary amylase gene family (*AMY1* genes). *AMYP1* is a pseudogene. *AMY1B* shows the greatest copy-number variability, ranging from 0 to 9 copies. A schematic of the gene cluster is shown underneath the heat map; 2B represents *AMY2B*, and so forth. **c** | Aggregate paralogue-specific copy number (psCN) genotypes of *AMY1* paralogues with estimates obtained by quantitative PCR (qPCR) directed at the three functional *AMY1* copies compared across 25 JPT individuals. These data show that the qPCR and read-depth data correlate. Data for part **b** and the *y* axis of part **c** are taken from REF. 75; data for the *x* axis of part **c** are taken from REF. 116.

between paralogous copies of duplicated gene families are necessary to better genotype these dynamic regions of the genome, which, in turn, is indispensible for understanding the phenotypic effect of duplications and their evolutionary importance.

Genotyping

Discovery techniques are complicated by the need to analyse data blind to the possible location of CNVs; stringent thresholds must therefore be applied to control false positives. By contrast, genotyping techniques offer increased power to detect CNVs once the variant is known, and more relaxed thresholds may be applied than for discovery[23]. Genotyping platforms need to assay fewer probes per locus than discovery technologies and thus can be performed at a reduced cost for a larger number of samples. In addition to sensitivity and specificity, the main considerations for genotyping are the number of target loci, cost and throughput. Genotyping of CNVs requires more than the accurate determination of zygosity. For example, the multicopy nature of duplicated regions requires phase determination such that a genotype of 2/3 versus 4/1 can be deduced from an absolute copy number of 5. Genotyping will be further enhanced by imputation from SNP data, and there is a pressing need to integrate SNP and CNV alleles. However, multicopy regions of the genome are often resistant to SNP imputation owing to recurrent mutations and difficulty in assigning SNP genotypes.

PCR-based techniques. Conventional PCR across sequenced breakpoints[59] and quantitative PCR provide the ability to rapidly and accurately screen a large number of samples at a very low cost per assay[94–96].

However, they are typically limited to a small number of loci. Multiplex ligation-dependent probe amplification (MLPA), multiplex amplifiable probe hybridization (MAPH) and multiplex amplicon quantification (MAQ) methodologies can simultaneously assay a larger number of loci (~40) on the basis of the quantification of PCR fragments in capillary electrophoresis experiments or microarray design[97–99]. These approaches require several optimization steps and are best applied when a large number of samples are to be analysed at a relatively low cost per sample. Alternatively, digital or single-molecule PCR allows screening of a large number of samples and sites in an emulsion or a microfluidic device. A significant benefit of this developing technology is that the enumeration of single-molecule PCR results, by real-time PCR or flow cytometry, allows the detection of events located on a single DNA fragment, which allows analysis of rare subpopulations or individual alleles[100–103].

SNP-array-based techniques. Another approach for the validation of a moderate number of loci in a large number of samples is the use of customizable SNP-based assays such as the Illumina BeadXpress system. Using the GoldenGate assay, in which labelled probes are ligated in the presence of a target sequence, the readout is similar to Illumina SNP arrays and, as such, copy-number states can be determined using allelic ratios. Experiments can be performed in a 96-well plate format that can interrogate up to 384 probes in a single well[104,105]. Adaption of this system through the development of specific algorithms for copy-number analysis and probe selection has demonstrated utility in the detection of rare (<1%) and common (>1%) CNVs with a low false discovery rate using five probes per region of interest[23,104,105]. Although the initial set-up cost for an assay can be high, the system can process thousands of samples at a low cost per sample, generating combined SNP and copy-number genotypes in a matter of weeks. An alternative approach is to use off-the-shelf or custom SNP platforms that contain tag SNPs, which allow imputation of the presence of a CNV. Although not all sites may be imputable, this approach demonstrates the potential for using genome-wide association study data to characterize common CNVs in large populations[32].

Array CGH-based techniques. Customized array CGH can be applied to genotype the largest number of CNV loci, as recently demonstrated by the Wellcome Trust Case Control Consortium (WTCCC)[32]. The WTCCC developed custom ~105,000 probe Agilent arrays covering 11,107 CNV loci discovered in various studies and applied this to a large population of ~19,000 samples; they successfully genotyped 4,000 CNVs. The same array was used by Conrad *et al.*[5] to validate 8,599 CNVs (of which they genotyped 5,238) from their ultra-high-density array calls in the HapMap data set. Both studies found that some regions were more readily genotyped than others, with deletions typically being easier to genotype than duplications or multiallelic loci; they also found that multiple normalization algorithms may be required for different loci[32]. Hence, targeted arrays share the same limitations as discovery arrays in the context of genomic regions that can be profiled. However, the cost-effectiveness of multiplex, targeted arrays for genotyping enables analysis of much larger populations than in a discovery context.

Sequencing-based approaches. As genome- and exome-sequencing data sets become routine, there has been a shift to genotyping using computational analysis of sequencing data. Several of the SV-detection algorithms mentioned above can accurately distinguish the homozygous versus heterozygous states of the discovered SVs[8,62,90]. However, genotyping common SVs across many genomes using discovery algorithms takes a substantial amount of time and resources. When the structural variants are known *a priori*, genotyping of such variants in larger cohorts can be performed quickly using methods specifically developed for the purpose. To this end, BreakSeq[106] was developed to build a library of SV breakpoints discovered in the literature, followed by validation using PCR. Raw reads from a newly sequenced genome were aligned to this breakpoint library to rapidly genotype the common structural variants. Kidd *et al.*[31] introduced a similar concept, termed diagnostic k-mer analysis, to genotype sequenced novel insertions using the NG-WGS data that can also discriminate heterozygous from homozygous insertions. Read-depth-based algorithms such as CopySeq[107] can genotype CNVs, and the SUN concept[75] described above can be used to genotype duplicated genome segments. Combining methods can improve accuracy in genotyping, as it can in discovery. Genome-STRiP, for example, considers read-pair, read-depth and split-read approaches but, importantly, puts this in the context of a population-genetics framework to substantially improve the sensitivity and specificity of deletion polymorphism genotyping[91]. These genotyping approaches quickly identify the existence (or lack of) common SVs in the genome of a human individual; however, most require an extensive database of structural variants sequenced at the breakpoint level[3–5,59,63,108].

An immediate benefit of NGS data sets is the potential to inform and improve existing experimental genotyping platforms, including SNP microarrays and array CGH[19,75] (FIG. 5). SNP microarray genotyping platforms, for example, assume a diploid state as the baseline copy for the population average and predict copy-number differences on the basis of that assumption. In addition, the underlying mosaic structure of several segments of the genome make the task of CNV detection using SNP microarrays more difficult. A comparison between sequence read-depth and SNP copy-number predictions reveals a discrepancy for 30% of genotype calls when the same DNA samples are compared[20,75]. Most of these discrepancies map to duplicated regions and, if adjusted by a defined integer, a near-perfect concordance is achieved. Thus, sequence read depth can be used to more accurately genotype copy number from SNP microarrays. Similarly, analysing the same samples using both read depth and array CGH can be used to calibrate copy

Tag SNP
A SNP in strong linkage disequilibrium with a set of SNPs or a copy number variant.

number for any specific region of the genome (FIG. 5b). Absolute copy-number estimates for duplicated genes, in conjunction with single-channel intensity data from array CGH experiments, allow for more accurate predictions of copy number on the basis of subtle changes in the dynamic range response. Thus, greater accuracy can be achieved in predicting copy number in duplicated regions using more affordable, high-throughput array CGH experiments. This reiterative complementarity of computational and experimental methodologies will be crucial to improving accuracy in detection and genotyping.

Future directions

With respect to the structural variation of the human genome, this is an exciting time for the field of human genetics. Significant advances have been made in understanding variation in the copy, content and structure of the human genome. Genotyping of structural variation is now possible on an unprecedented scale, and the past 2 years have seen discovery increase by orders of magnitude (BOX 2). The widespread application of affordable microarray hybridization-based approaches to thousands of normal and disease samples has provided glimpses of the landscape of larger CNVs[6,10,11,16,22]; we now appreciate that rare CNVs are collectively quite common in the general population and that the pattern of such variation is significantly different in individuals with neurocognitive and neuropsychiatric disease[109]. Here, the main bottlenecks are proving the pathogenicity of individual loci (which requires tens of thousands of unaffected individuals) and exploring smaller events (<25 kb) in disease populations. The application of NGS through read-depth, read-pair and split-read methods has expanded the spectrum of human genome structural variation to include tens of thousands of smaller events (>50 bp)[8]. More importantly, the specificity and dynamic range response of NGS provides unparalleled accuracy in terms of the content and absolute copy-number prediction[74,75]. As a result, the veil has been lifted on a large class of previously inaccessible genetic variants.

The most serious challenges that remain are the absence of a 'gold standard' for assessment of disparate discovery and genotyping methods, and the remaining biases in global discovery. There is no human genome published so far for which the complete spectrum of structural variation has been resolved. There is also no commercial platform than can claim to be comprehensive in terms of either genotyping or discovery. Different experimental methods and computational analyses of NGS data sets applied to the same human DNA samples show disappointingly low levels of overlap — a general trend that has persisted over years of SV-discovery effort (FIG. 2). The limitations of computational bandwidth notwithstanding, there is currently no suite of algorithms that could be applied to systematically resolve all classes of structural variants. Biases remain in terms of content, size and class, with most discovery efforts focused on deletions in unique sequences, as evidenced by the 1000 Genomes Project[8] and other published SV-discovery data sets (BOX 2).

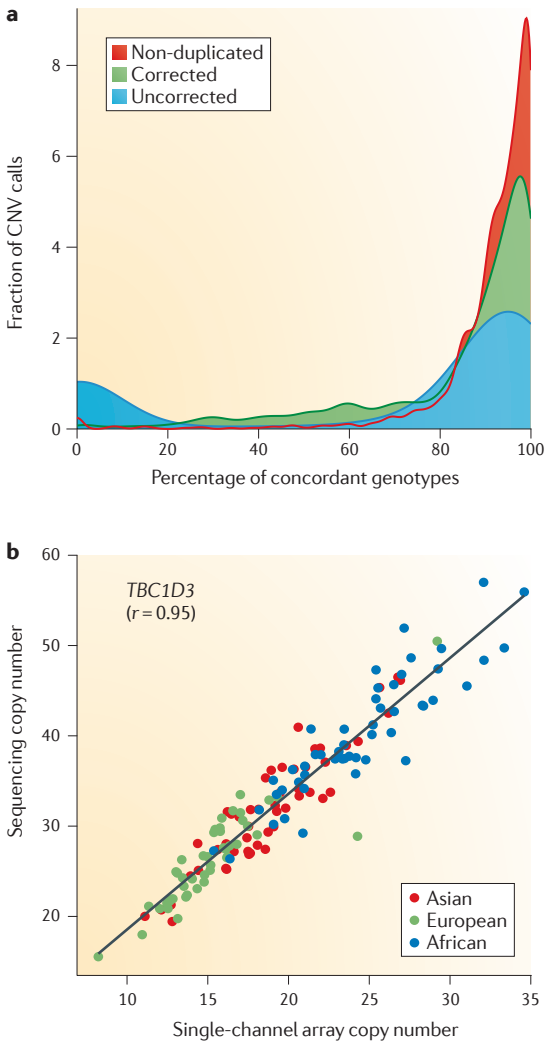


Figure 5 | **Improved copy number variant genotyping by the integration of computational and experimental approaches. a** | Absolute copy-number predictions made using sequence read depth[75] are compared to copy-number genotype calls made using SNP microarrays (Affymetrix 6.0)[20] on DNA from the same 114 individuals. The comparison shows good concordance in unique regions of the human genome (non-duplicated, red) when compared to all CNVs, including duplicated regions (uncorrected, blue). 94% of the discrepancies contain segmental duplications corresponding to 300 gene models. Analysis of the regions suggests population average copy numbers that differ from $n = 2$ (diploid). Readjusting the population average copy by an integer value using the read-depth estimations within the population ameliorates this bias (corrected, green) (change from 70% to 83% concordance). **b** | Single-channel array comparative genomic hybridization (array CGH) data (Agilent Technologies) is highly correlated with read-depth-based copy-number predictions for the highly duplicated *TBC1D3* gene family. This calibration with absolute copy-number prediction allows for a more accurate prediction of the copy number of duplicated regions for future array CGH experiments. Part **b** is modified, with permission, from REF. 75 © (2010) American Association for the Advancement of Science.

How can these deficiencies be overcome? Improvements in NGS technology that increase accuracy and read length would facilitate the discovery and genotyping of structural variation in more complex regions of the genome. The long-term goal should be the *de novo* assembly of human genomes to a standard comparable to or better than that of the current human reference genome (GRCh37). On the basis of the experiences of sequencing and finishing the first human genome, this will ideally require the sequencing of large molecules >100 kb in length with accuracies in excess of 99.9% — a feat beyond the reach of current third-generation platforms. An interim solution is the integration of computational and experimental methods. For example, the read-depth approach can be used to correct reference effects in array data[19,75]. Several experimental approaches (such as FISH and optical maps) and sequence analyses (such as read-depth and clone sequencing) are typically required to resolve the architecture of complex regions of the genome[50,110]. Massively parallel sequencing of large molecules[111] may provide an important step towards resolving the complete spectrum of structural variation, including balanced translocation and inversion events. As more sequence breakpoints become resolved for more difficult classes of structural variation, we will improve our ability to genotype using BreakSeq or diagnostic k-mer approaches[31,106]. Understanding copy, content and structure is an iterative process of evaluation that uses many orthogonal approaches and computational analyses. This effort requires committed investments from research laboratories, private industry and science funding agencies. Although getting to these answers will not be easy, the yield with respect to patterns of human genetic variation and insight into the architecture of human genetic disease will be worth the effort.

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Competing interests statement
E.E.E. declares competing financial interests: see Web version for details.

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