

DNA sequencing at 40: past, present and future

Jay Shendure^{1,2}, Shankar Balasubramanian^{3,4}, George M. Church⁵, Walter Gilbert⁶, Jane Rogers⁷, Jeffery A. Schloss⁸ & Robert H. Waterston¹

This review commemorates the 40th anniversary of DNA sequencing, a period in which we have already witnessed multiple technological revolutions and a growth in scale from a few kilobases to the first human genome, and now to millions of human and a myriad of other genomes. DNA sequencing has been extensively and creatively repurposed, including as a ‘counter’ for a vast range of molecular phenomena. We predict that in the long view of history, the impact of DNA sequencing will be on a par with that of the microscope.

DNA sequencing has two intertwined histories—that of the underlying technologies and that of the breadth of problems for which it has proven useful. Here we first review major developments in the history of DNA sequencing technologies (Fig. 1). Next we consider the trajectory of DNA sequencing applications (Fig. 2). Finally, we discuss the future of DNA sequencing.

History of DNA sequencing technologies

The development of DNA sequencing technologies has a rich history, with multiple paradigm shifts occurring within a few decades. Below, we review early efforts to sequence biopolymers, the invention of electrophoretic methods for DNA sequencing and their scaling to the Human Genome Project, and the emergence of second (massively parallel) and third (real-time, single-molecule) generation DNA sequencing. Some key technical milestones are also summarized in Box 1.

Early sequencing

Fred Sanger devoted his scientific life to the determination of primary sequence, believing that knowledge of the specific chemical structure of biological molecules was necessary for a deeper understanding¹. Ironically, given the state of sequencing technology for each biopolymer today, proteins and RNA came first.

The first protein sequence, of insulin, was determined in the early 1950s by Sanger, who fragmented its two chains, deciphered each fragment and overlapped the fragments to yield a complete sequence. His work showed unequivocally that proteins had defined patterns of amino acid residues². The later development of Edman degradation, a repeated elimination of an N-terminal residue from the peptide chain, made protein sequencing easier³. Although these methods were cumbersome, many proteins had been sequenced by the late 1960s, and it became clear that each protein's sequence varied across species and between individuals.

In the 1960s, RNA sequencing was tackled by this same general process: an RNA species was first fragmented with RNases, next the pieces were separated by chromatography and electrophoresis, then individual fragments were deciphered by sequential exonuclease digestion, and finally the sequence was deduced from the overlaps. The first RNA sequence, of alanine tRNA, required five people working three years with one gram of pure material (isolated from 140 kg of yeast) to determine 76 nucleotides⁴. This process was greatly simplified by ‘fingerprinting’ techniques, which included the separation of radioactively labelled RNA fragments and

visualization in two dimensions, with the resulting positions diagnostic of their size and sequence⁵.

The invention of DNA sequencing

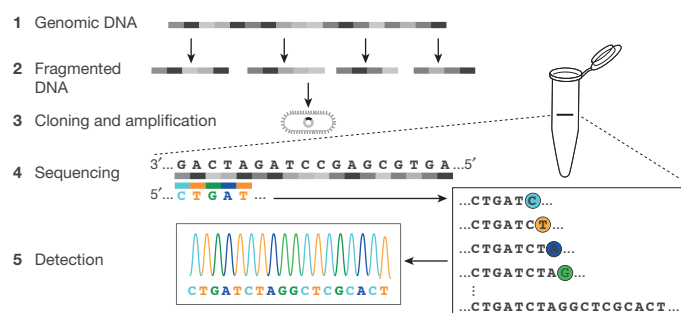
Early attempts to sequence DNA were cumbersome. In 1968, Wu reported the use of primer extension methods to determine 12 bases of the cohesive ends of bacteriophage lambda⁶. In 1973, Gilbert and Maxam reported 24 bases of the lactose-repressor binding site, by copying it into RNA and sequencing those fragments. This took two years: one base per month⁷.

The development, in around 1976, of two methods that could decode hundreds of bases in an afternoon transformed the field. Both methods—the chain terminator procedure developed by Sanger and Coulson, and the chemical cleavage procedure developed by Maxam and Gilbert—used distances along a DNA molecule from a radioactive label to positions occupied by each base to determine nucleotide order. Sanger's method involved four extensions of a labelled primer by DNA polymerase, each with trace amounts of one chain-terminating nucleotide, to produce fragments of different lengths⁸. Gilbert's method took a terminally labelled DNA-restriction fragment, and, in four reactions, used chemicals to create base-specific partial cleavages⁹. For both methods, the sizes of fragments present in each base-specific reaction were measured by electrophoresis on polyacrylamide slab gels¹⁰, which enabled separation of the DNA fragments by size with single-base resolution. The gels, with one lane per base, were put onto X-ray film, producing a ladder image from which the sequence could be read off immediately, going up the four lanes by size to infer the order of bases.

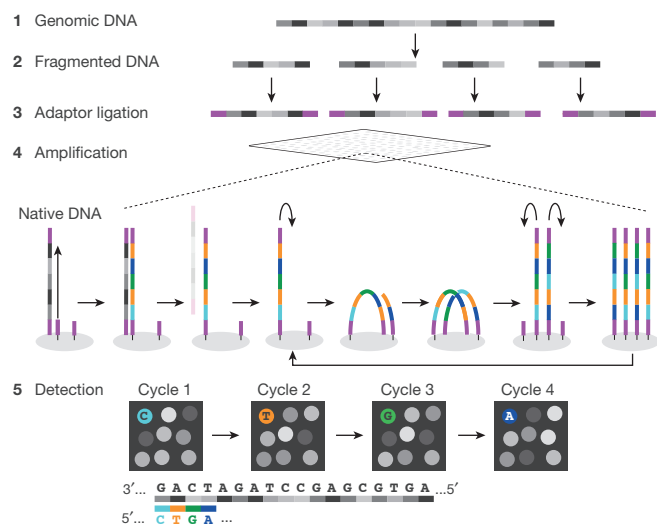
These methods came into immediate use. Shotgun sequencing—sequencing of random clones followed by sequence assembly based on the overlaps—was suggested by Staden in 1979¹¹, greatly facilitated by Messing's development of the single-stranded M13 phage cloning vector around 1980¹², and used to assemble genomes *de novo*, such as bacteriophage lambda as early as 1982¹³. By 1987, automated, fluorescence-based Sanger sequencing machines, developed by Smith, Hood and Applied Biosystems^{14,15}, could generate around 1,000 bases per day. Sequence data grew exponentially, approximating Moore's law and motivating the creation of central data repositories (such as GenBank) that, through search tools (such as BLAST¹⁶), amplified the value of each sequence and engendered a spirit of data sharing. By 1982, over half a million bases had been deposited in GenBank; by 1986, nearly 10 million bases (GenBank and WGS Statistics; <https://www.ncbi.nlm.nih.gov/genbank/statistics/>).

¹Department of Genome Sciences, University of Washington, Seattle, Washington, USA. ²Howard Hughes Medical Institute, Seattle, Washington, USA. ³Department of Chemistry, University of Cambridge, Cambridge, UK. ⁴Cancer Research UK Cambridge Institute, University of Cambridge, Cambridge, UK. ⁵The Wyss Institute & Department of Genetics, Harvard Medical School, Boston, Massachusetts, USA. ⁶Department of Molecular and Cellular Biology, Harvard University, Cambridge Massachusetts, USA. ⁷International Wheat Genome Sequencing Consortium, Little Eversden, Cambridge, UK. ⁸National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, USA.

First generation sequencing (Sanger)



Second generation sequencing (massively parallel)



Third generation sequencing (Real-time, single molecule)

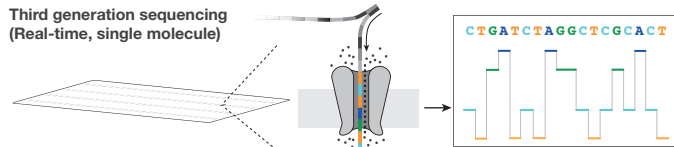


Figure 1 | DNA sequencing technologies. Schematic examples of first, second and third generation sequencing are shown. Second generation sequencing is also referred to as next-generation sequencing (NGS) in the text.

Scaling to the human genome

For the 'hierarchical shotgun' strategy that emerged as the workhorse of the Human Genome Project (HGP), large fragments of the human genome were cloned into bacterial artificial chromosomes (BACs). DNA from each BAC was fragmented, size-selected and sub-cloned. Individual clones were picked and grown, and the DNA was isolated. The purified DNA was used as a template for automated Sanger sequencing, the signal was extracted from laser-scanned images of the gels, and bases were called to finally produce the sequence. The fact that this process involved many independent steps, each of which had to work well, led sceptics to doubt it could ever be made efficient enough to sequence the human genome at any reasonable cost.

Indeed, as efforts to sequence larger genomes took shape, it became clear that the scale and efficiency of each step needed to be vastly increased. This was achieved in fits and spurts in the 1990s. Noteworthy improvements included: (1) a switch from dye-labelled primers to dye-labelled terminators, which allowed one rather than four sequencing reactions¹⁷; (2) a mutant T7 DNA polymerase that more readily incorporated dye-labelled terminators¹⁸; (3) linear amplification reactions, which greatly reduced template requirements and facilitated miniaturization¹⁹; (4) a magnetic bead-based DNA purification method that simplified automation of pre-sequencing steps²⁰; (5) methods enabling sequencing of double-stranded DNA, which enabled the use of plasmid clones and therefore paired-end

sequencing; (6) capillary electrophoresis, which eliminated the pouring and loading of gels, while also simplifying the extraction and interpretation of the fluorescent signal²¹; (7) adoption of industrial processes to maximize efficiencies and minimize errors (for example, automation, quality control, standard operating procedures, and so on).

Wet laboratory protocols were only half the challenge. Substantial effort was invested into the development of software to track clones, and into the interpretation and assembly of sequence data. For example, manual editing of the sequence reads was replaced by the development of phred, which introduced reliable quality metrics for base calls and helped sort out closely related repeat sequences²². Individual reads were then assembled from overlaps in a quality-aware fashion to generate long, continuous stretches of sequence. As more complex genomes were tackled, repetitive sequences were increasingly confounding. Even after deep shotgun sequencing of a BAC, some sequences were not represented, resulting in discontinuities that had to be tackled with other methods. Paired-end sequencing²³ helped to link contigs into gapped scaffolds that could be followed up by directed sequencing to close gaps. Some problems were only resolved by eye; scientists who were trained 'finishers' assessed the quality and signed off on the assembled sequence of individual clones²².

Although the process remained stable in its outlines, rapid-fire improvements led to steady declines in the cost throughout the 1990s, while parallel advances in computing increasingly replaced human decision making. By 2001, a small number of academic genome centres were operating automated production lines generating up to 10 million bases per day. Software for genome assembly matured both inside and outside of the HGP, with tools, such as phrap, the TIGR assembler and the Celera assembler, able to handle genomes of increasing complexity^{22,24,25}. A yearly doubling in capacity enabled the successful completion of high-quality genomes beginning with *Haemophilus influenza* (around 2 Mb, 1995) followed quickly by *Saccharomyces cerevisiae* (around 12 Mb, 1996) and *Caenorhabditis elegans* (around 100 Mb, 1998)^{26–28}. The HGP's human genome, which is 30 times the size of *C. elegans* and with much more repetitive content, came first as a draft (2001) and then as a finished sequence (2004)^{29,30}. The HGP was paralleled by a private effort to sequence a human genome by Craig Venter and Celera (2001)³¹ with a whole-genome shotgun strategy piloted on *Drosophila melanogaster* (around 175 Mb; 2000)³². The strategic contrasts between these projects are further discussed below.

By 2004, instruments were churning out 600–700 bp at a cost of US\$1 per read, but creating additional improvements was an increasingly marginal exercise. Furthermore, with the completion of the HGP, the future of large-scale DNA sequencing was unclear.

Massively parallel DNA sequencing

Throughout the 1980s and 1990s, several groups explored alternatives to electrophoretic sequencing. Although these efforts did not pay off until after the HGP, within a decade of its completion, 'massively parallel' or 'next-generation' DNA sequencing (NGS) almost completely superseded Sanger sequencing. NGS technologies sharply depart from electrophoretic sequencing in several ways, but the key change is multiplexing. Instead of one tube per reaction, a complex library of DNA templates is densely immobilized onto a two-dimensional surface, with all templates accessible to a single reagent volume. Rather than bacterial cloning, *in vitro* amplification generates copies of each template to be sequenced. Finally, instead of measuring fragment lengths, sequencing comprises cycles of biochemistry (for example, polymerase-mediated incorporation of fluorescently labelled nucleotides) and imaging, also known as 'sequencing-by-synthesis' (SBS).

Although amplification is not strictly necessary (for example, single-molecule SBS^{33–35}), the dense multiplexing of NGS, with millions to billions of immobilized templates, was largely enabled by clonal *in vitro* amplification. The simplest approach, termed 'colonies' or 'bridge amplification', involves amplifying a complex template library with primers immobilized on a surface, such that copies of each template remain tightly clustered^{36–39}. Alternatively, clonal PCR can be performed in an emulsion,

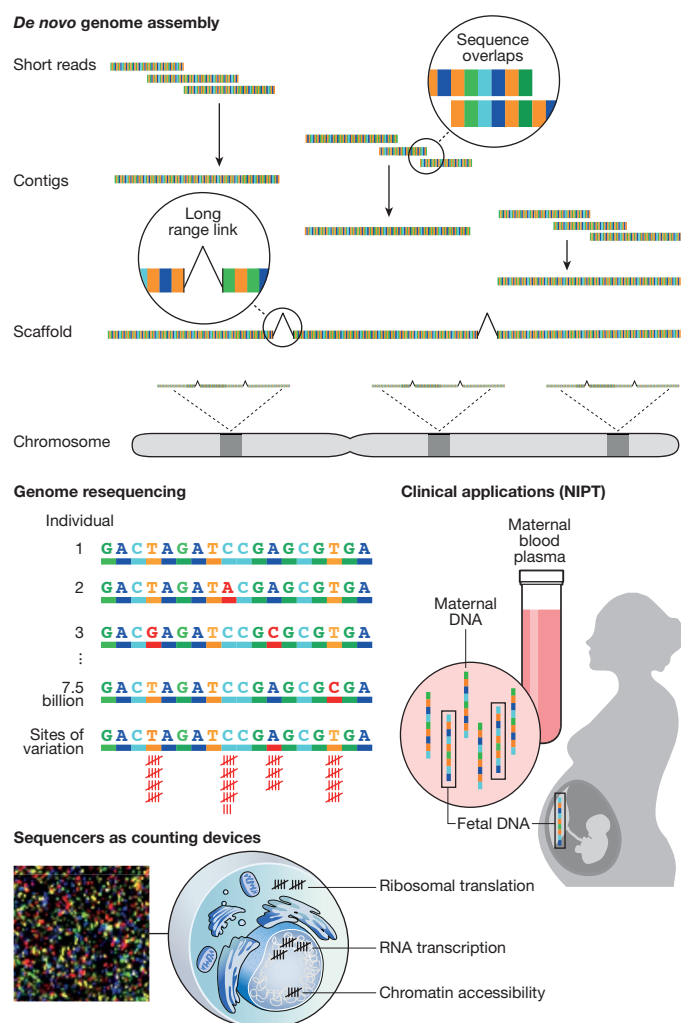


Figure 2 | DNA sequencing applications. Major categories of the application of DNA sequencing include *de novo* genome assembly, individual genome resequencing, clinical applications such as non-invasive prenatal testing, and using sequencers as counting devices for a broad range of biochemical or molecular phenomena.

such that copies of each template are immobilized on beads that are then arrayed on a surface for sequencing^{40–42}. A third approach involves rolling circle amplification in solution to generate clonal ‘nanoballs’ that are arrayed and sequenced⁴³.

For SBS, there were three main strategies. The pyrosequencing approach of Ronaghi and Nyren involves discrete, step-wise addition of each deoxynucleotide (dNTP). Incorporation of dNTPs releases pyrophosphate, which powers the generation of light by firefly luciferase⁴⁴. With an analogous approach, natural dNTP incorporations can be detected with an ion-sensitive field effect transistor^{45,46}. A second strategy uses the specificity of DNA ligases to attach fluorescent oligonucleotides to templates in a sequence-dependent manner^{41,43,47,48}. A third approach, which has proven the most durable, involves the stepwise, polymerase-mediated incorporation of fluorescently labelled deoxynucleotides^{33,34,49}. Critical to the success of polymerase-mediated SBS, was the development of reversibly terminating, reversibly fluorescent dNTPs, and a suitably engineered polymerase⁵⁰, such that each template incorporates one and only one dNTP on each cycle. After imaging to determine which of four colours was incorporated by each template on the surface, both blocking and fluorescent groups are removed to set up the next extension^{51–53}; this general approach was used by Solexa, founded by Balasubramanian and Klenerman in 1998.

The first integrated NGS platforms came in 2005, with resequencing of *Escherichia coli* by Shendure, Porreca, Mitra and Church⁴¹, *de novo*

assembly of *Mycoplasma genitalium* by Margulies, Rothberg and 454 (ref. 40), and resequencing of phiX174 and a human BAC by Solexa⁵⁴. These studies demonstrated how useful even very short reads are, given a reference genome to which to map them. Within three years, human genome resequencing would become practical on the Solexa platform with 35-bp paired reads⁵⁵.

In 2005, 454 released the first commercial NGS instrument. In the wake of the HGP, large-scale sequencing was still the provenance of a few genome centres. With 454 and other competing instruments that followed closely after, individual laboratories could instantly access the capacity of an entire HGP-era genome centre. This ‘democratization’ of sequencing capacity had a profound impact on the culture and composition of the genomics field, with new methods, results, genomes and other innovations arising from all corners.

In contrast to the monopoly of Applied Biosystems during the HGP, several companies, including 454 (acquired by Roche), Solexa (acquired by Illumina), Agencourt^{47,48} (acquired by Applied Biosystems), Helicos^{34,35} (founded by Quake), Complete Genomics⁴³ (founded by Drmanac) and Ion Torrent⁴⁶ (founded by Rothberg), intensely competed on NGS, resulting in a rapidly changing landscape with new instruments that were flashily introduced at the annual Advances in Genome Biology and Technology (AGBT) meeting in Marco Island, Florida. Between 2007 and 2012, the raw, per-base cost of DNA sequencing plummeted by four orders of magnitude⁵⁶.

Since 2012, the pace of improvement has slowed, as has the competition. The 454, SOLiD and Helicos platforms are no longer being developed, and the Illumina platform is dominant (although Complete Genomics⁴³ remains a potential competitor). Nonetheless, it is astonishing to consider how far we have come since the inception of NGS in 2005. Read lengths, although still shorter than Sanger sequencing, are in the low hundreds of bases, and mostly over 99.9% accurate. Over a billion independent reads, totalling a terabase of sequence, can be generated in two days by one graduate student on one instrument (Illumina NovaSeq) for a few thousand dollars. This exceeds the approximately 23 gigabases that were generated for the HGP’s draft human genome by a factor of 40.

Real-time, single-molecule sequencing

Nearly all of the aforementioned platforms require template amplification. However, the downsides of amplification include copying errors, sequence-dependent biases and information loss (for example, methylation), not to mention added time and complexity. In an ideal world, sequencing would be native, accurate and without read-length limitations. To reach this goal, stretching back to the 1980s, a handful of groups explored even more radical approaches than NGS. Many of these were dead ends, but at least two approaches were not, as these have recently given rise to real-time, single-molecule sequencing platforms that threaten to upend the field once again.

A first approach, initiated by Webb and Craighead and further developed by Korlach, Turner and Pacific Biosciences (PacBio), is to optically observe polymerase-mediated synthesis in real time^{57,58}. A zero mode waveguide, a hole less than half the wavelength of light, limits fluorescent excitation to a tiny volume within which a single polymerase and its template reside. Therefore, only fluorescently labelled nucleotides incorporated into the growing DNA chain emit signals of sufficient duration to be ‘called’. The engineered polymerase is highly processive; reads over 10 kb are typical, with some reads approaching 100 kb. The throughput of PacBio is still over an order of magnitude less than the highest-throughput NGS platforms, such as Illumina, but not so far from where NGS platforms were a few years ago. Error rates are very high (around 10%) but randomly distributed. PacBio’s combination of minimal bias (for example, tolerance of extreme GC content), random errors, long reads and redundant coverage can result in *de novo* assemblies of unparalleled quality with respect to accuracy and contiguity, for many species exceeding what would be possible even with efforts similar to the HGP.

A second approach is nanopore sequencing. This concept, which was first hypothesized in the 1980s^{59–61}, is based on the idea that patterns in

BOX 1

The milestones listed below correspond to key developments in the evolution of sequencing technologies. This is a large topic, and we apologize for any omissions.

Technical milestones

- 1953: Sequencing of insulin protein²
- 1965: Sequencing of alanine tRNA⁴
- 1968: Sequencing of cohesive ends of phage lambda DNA⁶
- 1977: Maxam–Gilbert sequencing⁹
- 1977: Sanger sequencing⁸
- 1981: Messing's M13 phage vector¹²
- 1986–1987: Fluorescent detection in electrophoretic sequencing^{14,15,17}
- 1987: Sequenase¹⁸
- 1988: Early example of sequencing by stepwise dNTP incorporation¹³⁹
- 1990: Paired-end sequencing²³
- 1992: Bodipy dyes¹⁴⁰
- 1993: *In vitro* RNA colonies³⁷
- 1996: Pyrosequencing⁴⁴
- 1999: *In vitro* DNA colonies in gels³⁸
- 2000: Massively parallel signature sequencing by ligation⁴⁷
- 2003: Emulsion PCR to generate *in vitro* DNA colonies on beads⁴²
- 2003: Single-molecule massively parallel sequencing-by-synthesis^{33,34}
- 2003: Zero-mode waveguides for single-molecule analysis⁵⁷
- 2003: Sequencing by synthesis of *in vitro* DNA colonies in gels⁴⁹
- 2005: Four-colour reversible terminators^{51–53}
- 2005: Sequencing by ligation of *in vitro* DNA colonies on beads⁴¹
- 2007: Large-scale targeted sequence capture^{93–96}
- 2010: Direct detection of DNA methylation during single-molecule sequencing⁶⁵
- 2010: Single-base resolution electron tunnelling through a solid-state detector¹⁴¹
- 2011: Semiconductor sequencing by proton detection¹⁴²
- 2012: Reduction to practice of nanopore sequencing^{143,144}
- 2012: Single-stranded library preparation method for ancient DNA¹⁴⁵

the flow of ions, which occur when a single-stranded DNA molecule passes through a narrow channel, will reveal the primary sequence of the strand. Decades of work were required to go from concept to reality. Firstly, electric field-driven transport of DNA through a nanometre-scale pore is so fast that the number of ions per nucleotide is insufficient to yield an adequate signal. Solutions have eventually been developed to these and other challenges, including interposing an enzyme as a 'ratchet', identifying and engineering improved nucleopore proteins, and better analytics of the resulting signals⁶². These advances recently culminated in successful nanopore sequencing, in both academia⁶³ and industry, most prominently by Oxford Nanopore Technologies (ONT), founded by Bayley in 2005. Sequence read lengths of ONT are on par with or exceed the reads generated by PacBio; with the longest obtained reads presently at 900 kilobases (ref. 64). A major differentiator from other sequencing technologies is the extreme portability of nanopore devices, which can be as small as a memory (USB) stick, because they rely on the detection of electronic, rather than optical, signals. Important challenges remain (for example, errors may not be randomly distributed), but progress is rapid.

Nucleic-acid sequencing would ideally also capture DNA modifications. Indeed, both PacBio and nanopore sequencing have demonstrated the detection of native covalent modifications, such as methylation^{64,65}. Single-molecule methods also open up the intriguing possibility of directly sequencing RNA^{66,67} or even proteins^{68–71}.

Since 1977, DNA-sequencing technology has evolved at a fast pace and the landscape continues to change shift under our feet. Although Illumina is presently the dominant supplier of sequencing instruments,

the commercial market is no longer monolithic and other technologies may successfully occupy important niches (for example, PacBio for *de novo* assembly and ONT for portable sequencing). Neither NGS nor single-molecule methods have fully plateaued in cost and throughput, and there are additional concepts that are still in development, which are not discussed here (for example, solid-state pores and electron microscopy)^{70,71}. Not all will work out, but as the above examples make clear, transformative sequencing technologies can take decades to mature.

Applications of DNA sequencing

The range and scope of DNA sequencing applications has also expanded over the past few decades, shaped in part by the evolving constraints of sequencing technologies. Below we review key areas of application including *de novo* genome assembly, individual genome resequencing, sequencing in the clinic and the transformation of sequencers into molecular counting devices. Some key milestones for the generation of reference genomes and development of applications and software are summarized in Box 2.

***De novo* genome assembly**

For its first 25 years, the primary purpose of DNA sequencing was the partial or complete sequencing of genomes. Indeed, the inception of Sanger sequencing in 1977 included the first genome (phiX174; 5.4 kb), essentially assembled by hand⁷². However, DNA sequencing was only one of several technologies that enabled assembly of larger genomes. If the DNA sequence was random, arbitrarily large genomes could be assembled to completion solely based on fragment overlaps. However, it is not random, and the combination of repetitive sequences and technical biases makes it impossible to obtain high-quality assemblies of large genomes from kilobase-scale reads alone. Additional 'contiguity information' is required.

For the HGP^{29,30}, these additional sources of contiguity information included the following. (1) Genetic maps, which were based on the segregation of genetic polymorphisms through pedigrees, that provided orthogonal information about the order of sequences locally and at the scale of chromosomes. (2) Physical maps, for which BACs were cloned, restriction-enzyme 'fingerprinted' to identify overlaps and ordered into a 'tiling path' that spanned the genome. Clones were individually shotgun sequenced and assembled, thereby isolating different repeat copies from one another, and then further ordered and assembled. (3) Paired-end sequencing, introduced by Ansorge in 1990²³, comprises sequencing into both ends of a DNA fragment of approximately known length, effectively linking those end-sequences. Depending on the cloning method, the spanned length could range from a few kilobases to a few hundred kilobases. Sequence coverage at 8–10-fold redundancy, coupled to these sources of contiguity information, enabled not only genome assembly, but also improved quality to about 1 error per 100,000 bases for most of the genome. Additional, focused experiments were performed to fill the gaps or clarify ambiguities.

The Celera effort went straight to paired-end sequencing, eschewing physical maps as an intermediate³¹. An important advance was the transition from greedy algorithms, such as phrap and the TIGR assembler, to the Celera assembler's graph-based approach (overlap–layout–consensus)^{22,24,25}. Although Celera had a reasonable strategy for a draft genome, because of the pervasiveness of repetitive sequences, it did not, by itself, result in a high-quality reference, such as the one produced by the HGP's clone-based approach. The current human reference genome descends from the HGP's 2004 product³⁰, with continuous work by the Genome Reference Consortium to further improve it, including regular releases of reference genome updates⁷³.

With the advent of NGS in 2005, the number of *de novo* assemblies increased vastly. The seemingly disastrous combination of short reads and repetitive genomes was overcome by new assembly algorithms based on de Bruijn graphs (for example, EULER and Velvet)^{74,75}. Nonetheless, particularly when applied to larger genomes and when compared to the genomes of the HGP, their quality was, on average, quite poor. Although shorter read lengths are partly to blame, this is usually overstated. Instead, a principal reason for the poorer quality was the paucity of contiguity

BOX 2

The milestones listed below correspond to key developments in the availability of new reference genomes, new sequencing-related computational tools and the applications of DNA sequencing in new ways or to new areas. These are large topics, and we apologize for any omissions.

Genome milestones

1977: Bacteriophage Φ X174 (ref. 72)

1982: Bacteriophage lambda¹³

1995: *Haemophilus influenzae*²⁶

1996: *Saccharomyces cerevisiae*²⁷

1998: *Caenorhabditis elegans*²⁸

2000: *Drosophila melanogaster*³²

2000: *Arabidopsis thaliana*¹⁴⁶

2001: *Homo sapiens*^{29–31}

2002: *Mus musculus*¹⁴⁷

2004: *Rattus norvegicus*¹⁴⁸

2005: *Pan troglodytes*¹⁴⁹

2005: *Oryza sativa*¹⁵⁰

2007: *Cyanidioschyzon merolae*¹²⁶

2009: *Zea mays*¹⁵¹

2010: Neanderthal⁸⁸

2012: Denisovan¹⁴⁵

2013: The HeLa cell line^{152,153}

2013: *Danio rerio*¹⁵⁴

2017: *Xenopus laevis*¹⁵⁵

Computational milestones

1981: Smith–Waterman¹⁵⁶

1982: GenBank (<https://www.ncbi.nlm.nih.gov/genbank/statistics/>)

1990: BLAST¹⁶

1995: TIGR assembler²⁴

1996: RepeatMasker

1997: GENSCAN¹⁵⁷

1998: phred, phrap, consed²²

2000: Celera assembler²⁵

2001: Bioconductor

2001: EULER⁷⁴

2002: BLAT¹⁵⁸

2002: UCSC Genome Browser¹⁵⁹

2002: Ensembl¹⁶⁰

2005: Galaxy¹⁶¹

2007: NCBI Short Read Archive

2008: ALLPATHS¹⁶²

2008: Velvet⁷⁵

2009: Bowtie⁸³

2009: BWA⁸²

2009: SAMtools⁸⁴

2009: BreakDancer¹⁶³

2009: Pindel¹⁶⁴

2009: TopHat¹¹⁵

2010: SOAPdenovo¹⁶⁵

2010: GATK⁸⁵

2010: Cufflinks¹¹⁶

2011: Integrated Genomics Viewer¹⁶⁶

2013: HGAP/Quiver¹⁶⁷

2017: Canu⁸¹

Application milestones

1977: Genome sequencing⁷²

1982: Shotgun sequencing¹³

1983, 1991: Expressed sequence tags^{107,108}

1995: Serial analysis of gene expression¹⁰⁹

1998: Large-scale human SNP discovery¹⁶⁸

2004: Metagenome assembly¹²²

2005: Bacterial genome resequencing with NGS^{40,41}

2007: Chromatin immunoprecipitation followed by sequencing (ChIP–seq) using NGS¹¹⁷

2007–2008: Human genome and cancer genome resequencing using NGS^{55,90–92}

2008: RNA-seq using NGS^{110–114}

2008: Chromatin accessibility using NGS¹¹⁸

2009: Exome resequencing using NGS⁹⁷

2009: Ribosome profiling using NGS¹¹⁹

2010: Completion of Phase I of the 1000 Genomes Project⁹⁸

2010: *De novo* assembly of a large genome from short reads¹⁶⁹

2011: Haplotype-resolved human genome resequencing using NGS^{170,171}

2016: Human genome *de novo* assembly with PacBio¹⁷²

2017: Human genome *de novo* assembly with nanopore⁶⁴

methods to complement NGS. Paired-end sequencing was possible with NGS, but *in vitro* library methods were more restricted with respect to the distances that could be spanned. Furthermore, the field lacked ‘massively parallel’ equivalents of genetic and physical maps.

This ‘dark’ period notwithstanding, there are good reasons to be optimistic about the future of *de novo* assembly. Firstly, *in vitro* methods that subsample high molecular weight (HMW) genomic fragments, analogous to hierarchical shotgun sequencing, have recently been developed^{76,77}. Secondly, methods, such as Hi-C (genome-wide chromosome conformation capture) and optical mapping, provide scalable, cost-effective means of scaffolding genomes into chromosome-scale assemblies^{78–80}. Finally, the read lengths of PacBio and ONT sequencing have risen to hundreds of kilobases, and are now more limited by the preparation of HMW DNA than by the sequencing itself. The absence of cloning or amplification steps in single-molecule sequencing pays off, as shown by high-quality PacBio *de novo* assemblies of bacterial genomes with extreme GC content. Long reads have resulted in a re-emergence of strategies used by the Celera assembler, improved to deal with the high error rates and multiple platforms⁸¹. By combining long reads and even longer-range contiguity information (for example, subsampling HMW DNA, chromatin proximity, optical maps and so on), *de novo* genome assemblies of the quality of the original human reference genome using ‘post-Sanger’ approaches are finally within sight^{73,80}.

Genome resequencing

After the HGP, a clear next step was to catalogue genetic variation among humans, that is, ‘resequencing’. Because Sanger sequencing costs remained high, resequencing was primarily used to discover common variants, which were then cost-effectively genotyped with microarrays to facilitate genome-wide association studies. The rallying cry for changing this was the ‘US\$1,000 human genome’, the ambitious goal of the resequencing of individuals at a cost nearly one-million-fold below that of assembling the first human genome. The US\$1,000 genome concept was discussed as early as 2001 (at the University of California, Santa Cruz Human Genome Symposium (<http://genomesymposium.ucsc.edu>)), when NGS strategies barely existed, and was formalized a few years later by the Revolutionary DNA Sequencing Technologies program of the National Human Genome Research Institute (NHGRI). The commitment of US\$220 million in funding to over 40 academic and 27 commercial entities has helped to drive much of the technological development described above, including direct or indirect support of nearly every successful commercial platform.

Resequencing, that is, mapping sequence reads to a reference genome to identify genetic variants, is a very different task than genome assembly. New algorithms, such as Bowtie and Burrows–Wheeler Aligner (BWA), borrowed concepts from data-compression techniques to enable millions of reads to be efficiently mapped to the reference genome^{82,83}. Redundant coverage (for example, 30-fold) is necessary to identify heterozygous

variants as well as to distinguish sequencing errors from bona fide variants. Popular packages, initially SAMtools and later GATK, adapted the confidence framework of phred to NGS bases, reads and variants^{84,85}. Short reads, particularly when paired, can be uniquely mapped to most of the human genome. But most is not all, and a problem of short-read resequencing is that variants in repetitive regions and structural variants are routinely missed. The extent of this shortcoming is quantified by recent studies that resequence human genomes with PacBio⁸⁶. A second aspect of incompleteness relates to phase relationships between variants in a diploid genome, that is, haplotypes⁸⁷. Fortunately, haplotypes are recovered by many of the same methods that enable contiguity for *de novo* NGS assemblies (and ideally, even *de novo* assemblies would be haplotype-resolved)⁷⁷. Although still not broadly used, these methods are becoming increasingly scalable.

The HGP's human genome was constructed from a mosaic of DNA donors, but mostly derives from one individual, from Buffalo, New York, who had roughly equal parts European and African ancestry⁸⁸. The first individual to have their whole genome resequenced was Craig Venter in 2007, one of the subjects of the Celera genome, which was supplemented with additional data⁸⁹. This was quickly followed in 2008 by the genome of Jim Watson on 454 (ref. 90), and then the genomes of two anonymous individuals^{55,91} and the germline and tumour genome of a patient⁹² on Solexa/Illumina, and five individuals on Complete Genomics⁴³. In this period, whole-genome sequencing (WGS) remained too expensive for most groups to scale, motivating the development of targeted capture methods^{93–96} and then whole-exome sequencing (WES), that is, selective sequencing of the 1–2% of the genome that encodes proteins⁹⁷.

As costs approached US\$1,000 for WGS⁵⁶ and a few hundred dollars for WES, the pace at which individual humans are resequenced has accelerated. The 1000 Genomes Project, launched in 2008, released low-coverage WGS of a few hundred individuals in 2010 and a few thousand individuals in 2015^{98,99}. The Exome Sequencing Project released over 6,500 exomes in 2013¹⁰⁰. The recently released Genome Aggregation Database (<http://gnomad.broadinstitute.org/>) includes more than 120,000 exomes and over 15,000 genomes. The Genomics England (<https://www.genomicsengland.co.uk/>), GenomeAsia100K (<http://www.genomeasia100k.com/>) and NHLBI TOPMed (Trans-Omics for Precision Medicine, <https://www.nhlbiwgs.org/>) projects each aim to complete WGS on more than 100,000 individuals within the next year or two. Given that these projects represent a fraction of all sequencing being conducted, it is plausible that the genomes of over one million humans have already been resequenced by WES or WGS.

Clinical applications of sequencing

Our ability to sequence human genomes has vastly outpaced our ability to interpret genetic variation, which partly explains why clinical medicine has yet to wholeheartedly embrace WGS. Nonetheless, there are some areas in which DNA sequencing is already proving clinically useful, three of which we highlight here.

The most unexpected area of the clinical impact of DNA sequencing has been non-invasive prenatal testing (NIPT, see Fig. 2). Pioneering studies by Lo and Quake in 2008 have demonstrated that the simple counting of DNA fragments released into the maternal circulation by a fetus during pregnancy can detect chromosomal aneuploidies^{101,102}. Screening tests that were based on this strategy were adopted faster than any molecular test in history, and several million pregnant women around the world have already benefited from low-pass WGS for NIPT.

An early application of WES was to rapidly discover new genes for, and to diagnose patients affected by, Mendelian disorders^{97,103}. This was quickly followed by the discovery that substantial proportions of neurodevelopmental disorders are attributable to *de novo* mutations in coding sequences¹⁰⁴. WES is increasingly used as a primary tool for diagnosing Mendelian and neurodevelopmental disorders, particularly in paediatric populations, with the rate of diagnosis of patients with suspected Mendelian disease by WES at 25% and rising¹⁰⁵.

Our understanding of cancer, fundamentally a disease of the genome, is gradually being transformed by DNA sequencing. Large-scale resequencing has laid bare the extraordinary genetic heterogeneity of cancers, effectively defining a molecular taxonomy¹⁰⁶. DNA sequencing is impacting clinical cancer care by: (1) suggesting targeted therapies, based on the mutations present in an individual cancer; (2) enabling non-invasive diagnosis or monitoring by sequencing of tumour-released circulating cells or cell-free DNA; (3) identifying cancer-specific, protein-altering mutations that may serve as neoantigens for 'personal vaccines'. Although, the success stories in each of these areas are still few and far between, relative to the overall burden of cancer, progress is clearly being made.

Sequencers as a molecule counting device

While 'expressed sequenced tags'¹⁰⁷ were considered a shortcut to gene discovery as early as 1983¹⁰⁸, it was SAGE (serial analysis of gene expression; 1995) that introduced the idea of sequencing to 'digitally quantify' gene expression¹⁰⁹. SAGE concatenated cDNA-derived tags for Sanger sequencing, with tags that are just long enough to map to a gene. As early as 2000, Brenner and Lynx Therapeutics demonstrated 'massively parallel signature sequencing' of cDNA tags, an important forerunner of NGS⁴⁷. However, this concept was not widely adopted until the development of RNA sequencing (RNA-seq) by five groups in 2008. RNA-seq uses NGS to quantify and characterize the transcriptome by shotgun sequencing of either full-length or 3' ends of cDNA^{110–114}. RNA-seq has marked advantages over microarrays, the most notable of which is that transcript counts lead to straightforward statistics relative to analogue, hybridization-based signals, facilitated by new software packages, such as TopHat and Cufflinks^{115,116}.

Also around 2008, small laboratories that were early adopters of NGS developed 'digital quantification' methods for transcription-factor binding¹¹⁷, chromatin accessibility¹¹⁸ and translation¹¹⁹. In the following decade, hundreds of protocols were developed that facilitate the use of DNA sequencing as a 'molecule counter' for the characterization of a remarkable range of biochemical or molecular phenomena, including transcription, translation, DNA replication, the secondary structure of RNA, chromosome conformation, nucleic-acid modifications, post-translational modifications, nucleic acid–protein interactions and protein–protein interactions. These are catalogued in other reviews and resources (ref. 120 and <http://enseqlopedia.com/>).

The use of sequencers as molecule-counting devices was immediately immensely popular, and probably had a larger role than assembly or resequencing in driving the widespread adoption of NGS in biomedical research. DNA sequencers are increasingly to the molecular biologist what a microscope is to the cellular biologist—a basic and essential tool for making measurements. In the long run, this may prove to be greatest impact of DNA sequencing.

Metagenome sequencing

Shotgun sequencing of complex communities of microorganisms^{121–123}, for example, metagenome sequencing of environmental or human microbiomes, has emerged as a field of its own, bringing with it unique challenges with respect to assembly, resequencing and counting. Other reviews have recently covered this topic^{124,125}.

The future of DNA sequencing

In the long view of scientific history, DNA sequencing remains a young technology. Here, we briefly consider its future in a few existing or emerging areas.

Genome diversity

A 100% complete genome, that is, the telomere-to-telomere sequence for each chromosome with no gaps or ambiguities, has been achieved for possibly only one eukaryote so far¹²⁶. As sequencing technologies continue to evolve, we are optimistic that we will resolve challenging regions of additional genomes (for example, centromeres). There are

millions of living species on earth (and far more extinct species), each with a genome waiting to be sequenced, as well as countless microbiomes and metagenomes. A comprehensive view of genomic diversity may prove useful in surprising ways, for example, for protein structure determination¹²⁷.

Population-scale resequencing

We are approaching the milestone where approximately 0.1% of living humans will have had their genomes resequenced to some degree, while resequencing of the genomes of our ancestors and other hominins is reshaping our understanding of human history⁸⁸. The number of *de novo* point mutations occurring in recent generations vastly exceeds the number of nucleotides in the human genome. Eventually, aggregating tens of millions of genomes may enable a nucleotide-level footprint of the human genome (that is, observing all heterozygous variants compatible with life). DNA sequencing also is increasingly useful for forensics, without necessarily requiring a sample from the identified individual¹²⁸.

Developmental biology

We each develop from a single cell into a highly organized mass of trillions of cells. However, our understanding of development remains coarse. Recent technologies enable scalable, sequencing-based profiling of single cells. Although popular approaches are *ex vivo* (for example, single-cell RNA-seq), a more radical approach is to perform RNA or protein sequencing *in situ*, thereby retaining the spatial context^{129,130}. Other emerging strategies use *in vivo* genome editing to track cell-lineage relationships¹³¹ or transport barcodes to catalogue neuronal connections¹³². Editing of DNA can potentially be used to record biological events more generally, for example, to monitor gene expression¹³³ or calcium¹³⁴.

Real-time, portable sensors

Nanopore sequencers currently have a mass of 70 g and yield data within 30 min of sample application. One can imagine disseminated networks of nanopore sequencers enabling 'universal monitoring' of nucleic acids, in environmental settings and in everyday human life, for example, fine-grained tracking of our air, food and body, potentially streaming data from millions of devices and integrating with GPS and audio-visual data.

Unconventional uses

DNA-sequencing technologies will probably prove useful in additional, surprising ways. For example, NGS has recently been used to recover large amounts of data encoded in synthetic DNA¹³⁵. Nanopores may find uses beyond sequencing, for example, for monitoring analyte binding¹³⁶, chemical nanomachines¹³⁷ or protein folding/unfolding¹³⁸.

DNA sequencing as the new microscope

It has been about 400 years since the invention of light microscopy, a technology which continues to be used and to evolve. By comparison, it has been only 40 years since the invention of DNA sequencing; the technologies for which are likely to also continue to develop in the coming decades and centuries. On the basis of how quickly it has transformed biomedical research, and is beginning to transform clinical medicine, we predict that DNA sequencing will have a longevity and impact on par with or exceeding that of the microscope.

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CORRECTIONS & AMENDMENTS

CORRECTION

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Publisher Correction: DNA sequencing at 40: past, present and future

Jay Shendure, Shankar Balasubramanian, George M. Church, Walter Gilbert, Jane Rogers, Jeffery A. Schloss & Robert H. Waterston

Correction to: *Nature* <https://doi.org/10.1038/nature24286>, published online 11 October 2017.

In this Review, the year of publication of reference 54 should be 2005, not 2015. In Box 2, “1982: GenBank (<https://www.ncbi.nlm.nih.gov/genbank/statistics/>)” should read “1982: Genbank/ENA/DDBJ” and “2007: NCBI Short Read Archive” should read “2007: NCBI and ENA Short Read Archives”; this is because the launches of these American, European and Japanese databases were coordinated. These errors have not been corrected.