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Yusuf Deeni
School of Science, Engineering and Technology, Abertay University, Dundee, Scotland.

Tommaso Beccari
Department of Pharmaceutical Sciences, University of Perugia, Italy.

Munis Dundar
School of Medicine, Erciyes University, Kayseri, Turkey

Jill S. Gartland
Life Sciences, Glasgow Caledonian University, Glasgow, Scotland.

Michele Maffia
Department of Biological and Environmental Science and Technology, University of Salento, Lecce, Italy.

Mariapia Viola-Magni
Fondazione Enrico Puccinelli, Ponte S. Giovanni, Perugia, Italy.

Kevan M.A. Gartland
Life Sciences, Glasgow Caledonian University, Glasgow, Scotland.

Correspondence:
Kevan M.A. Gartland
Life Sciences, Glasgow Caledonian University, Glasgow, Scotland.

Novel technologies and their applications in biotechnology

Yusuf Deeni, Tommaso Beccari, Munis Dundar, Jill S. Gartland, Michele Maffia, Mariapia Viola-Magni, Kevan M.A. Gartland

Abstract
Advances in technology are rapidly improving the range of available biotechnological tools and applications. Interfacial opportunities, such as those combining biomedical improvements with aspects of engineering, computing and innovative biomaterials are at the forefront of these enhancements. Personalised and predictive screening, pre-disease and biomarker identification, gene therapy and genome editing as well as large scale analysis of data from single cell sequencing and digital PCR screening programmes are being used in new approaches to earlier disease detection, rapid point of care diagnostics, increase understanding and improve outcomes. Multi-disciplinary approaches are benefitting all areas of biotechnology, from synthetic biology to microbial community dynamics and new product development, as innovative applications combine elements of novel technologies to address previously intractable problems. These 'translational biotechnology' applications combine ingenuity, creativity and complexity by building upon the torrent of molecular and cellular data available and will benefit mankind, society and the environment for decades to come.

Keywords: Biotechnology, biomedicine, bioinformatics, sequencing, genomics, 3d-printing, biomarkers, bionanomaterials

1. Introduction

Technologies, interfaces and creativity

Technologies are capable of transforming activities by making things easier, or simpler to achieve. Biotechnological applications of novel technologies include improving the effectiveness or efficiency of processes or activities, with ultimate benefits for health and well-being, quality of life, environmental or economic performance. The interfaces between emergent and existing technologies provide some of the most exciting opportunities for biotechnological exploitation. Realising the potential of new and emergent technologies requires imaginative use of creativity to translate technologies into improved processes, activities or understanding. Translating biotechnological innovations into successful improvements can take place at a range of levels, from commercial companies to the activities of individual citizens. In this work, a range of novel, frequently interfacial technologies with biotechnological applications are discussed and the outlook for future enhancements considered. The translational biotechnology applications considered range from the level of individual nucleotides to whole organism engineering and from 3-dimensional printing to wearable devices. Each of these applications benefits from the imaginative use of novel or emergent technologies to address new or existing questions in new ways. Improving the efficiency of activities or processes by combining elements from a range of disciplines, frequently involves using information technology advances to realise the potential offered by interdisciplinary and creative use of novel technologies.

2. Sequencing advances and applications

The torrent of technologies to obtain new nucleic acids sequence information has led to important questions about what to do with all of this information, producing an explosion of new bioinformatics and data mining developments. Methods for sequencing from individual single cells now allow a variety of functional cell states to be investigated, following developmental, temporal or disease related changes in genomes and gene expression in ways not previously possible.

This has major implications for stratified and personalised medicine, both in terms of identifying appropriate therapeutic doses and responses to documentable changes in disease state, but also in terms of predictive and preventative indicators of future conditions. Identifying disease producing genetic variants is an important tool in understanding different disease states [1]. The dramatic falls in the cost of whole exome sequencing, or even whole genome sequencing, allied to the universal availability of computing tools to analyse this information, are continuously adding to our analytical powers in this rapidly changing field. Optical mapping approaches for example, have been used to overcome previous limitations of assembling thousands of relatively short fragments, in sequencing the 2.66 Gb goat genome [2]. Single DNA molecules cleaved by restriction enzymes are imaged, rapidly generating whole genome maps, from which super-scaffolds can be generated, allowing commercial scale sequencing of large, complex genomes [3]. Combining sequencing advances and bioinformatics can enhance clinical studies through the development and validation of cancer genomic profiling tests, based on massively parallel DNA sequencing. Using formalin-fixed and paraffin-embedded clinical specimens, nucleotide substitutions, indels, copy number alterations and fusions have been assessed for 287 cancer-related genes [4], achieving test sensitivities of 95-99%. By assessing 2,221 clinical cases actionable genomic alterations were detected in 76% of tumours, representing a threefold increased effectiveness over current diagnostic tests. More accurate identification of genomic changes can only benefit diagnosis and the evaluation of therapy options tailored to individual tumour conditions.

Although primarily driven by the high value biomedical arena, the tools, insights and understandings developed as a result of advances in 'Next Generation' sequencing and bioinformatics will undoubtedly cascade to all areas of the life sciences. This will require new approaches to databasing sequence variant information, allowing the construction of genetic disease data ecosystems for key conditions, and greater integration of sequence data with pharmacogenetic insights in personal or family-based analyses, allowing individually tailored treatment options to be identified, with a greater probability of a positive outcome [1].

Single-cell genome and transcriptome sequencing methods are generating a fresh wave of biological insights into development, cancer and neuroscience. Genome and transcriptome sequencing require more starting material than the few picograms found in an individual cell, pushing the limits of amplification technology. Heavy amplification also propagates errors and biases, leading to uneven coverage, noise and inaccurate quantification. Recent technical advances have helped mitigate these challenges, making single-cell sequencing an appealing way to address an expanding set of problems. Rare cell types, heterogeneous samples, phenotypes associated with mosaicism or variability, and microbes that cannot be cultured are good candidates for single-cell approaches. Single-cell sequencing can enable the discovery of clonal mutations, cryptic cell types or transcriptional features that would be diluted or averaged out in bulk tissue studies [5].

Single-cell genome analysis is now influencing areas as diverse as microbial ecology, cancer, prenatal genetic diagnosis and the study of human genome structure and variation [6, 7]. Several important considerations influence the quality of data generated from a single cell. Of particular note is the inevitable problem that the transcriptome will change in response to manipulation, which is likely to be more acute in individual cells. With this consideration in mind, single-cell transcriptome data should be interpreted partially as a perturbation experiment until less disruptive RNA isolation methods can be developed. Isolation of single cells is the technique that is arguably in greatest need of development and standardization. Using a patch pipette or nanotube to harvest the cytoplasmic contents of single cells is a common method for the isolation of cellular RNA, but it may leave cellular sub-compartments behind. The classic constraint of single-cell approaches is the need for substantial amplification, which may misrepresent the original DNA sequence or RNA population. The problem is especially acute when working with DNA, where only a single molecule is available. For DNA, the main problem is coverage. Extensive PCR-based amplification may yield higher coverage, but this is typically at the expense of uneven representation and error amplification [8].

In 2012, Xie's group described a new strategy called MALBAC, or multiple annealing and looping-based amplification cycles, that involves five cycles of multiple displacement amplification (MDA) as a kind of preamplification during which newly amplified fragments form closed loops [5]. MALBAC sequencing combines elements of MDA and conventional PCR, to try and reduce bias in whole genome amplification methods used previously [9]. Building upon reductions in amplification bias and amplicons of more than 12 kb in length achieved through MDA [10], MALBAC uses primers consisting of a common 27 nucleotide tag and eight random nucleotides to anneal to template DNA for strand displacement synthesis, generating partial amplicons which in turn act as template for wider genome coverage reducing amplification bias. Following five cycles of quasilinear amplification, looped full amplicons are subjected to 20 PCR cycles and sequenced. MALBAC has been used to increase the efficiency of detecting both alleles of known single nucleotide variations seven-fold, whilst reducing amplification bias, as compared to MDA alone[11]. Although MALBAC can generate false positives in single nucleotide allelic identifications, further optimisation is likely to generate a wave of new applications for the identification and analysis of single nucleotide level changes in the genomes of single cells. The data obtained will provide useful insights into diseased states, predictive diagnostics, genome structure and population variations [6, 7].

At the level of individual cells, all diseases show heterogeneity in their pathology. Single-cell studies may lead to a better understanding of why some cells degenerate while adjacent cells are normal, or why some cells are drug responsive but others are not [12]. In this scenario cancer research stands to benefit enormously from single-cell sequencing approaches. Cancer cells often undergo high mutation rates, and tumours tend to be heterogeneous. Identifying which subsets of cells, called clones, are present and evolve into metastases or respond in a certain way to chemotherapy is critical to understanding and fighting the disease. In particular, circulating tumour cells (CTCs), which break off from a tumour and seed metastasis, are those rare cells whose genomes or transcriptomes might offer clues for diagnosis, monitoring or treatment.

3. Biomarker analysis

Biomarker analysis uses changes in key data indicators as predictive, preventative or personalised tools for disease states, for example using miRNA to detect lung tumours earlier than

conventional screening methods. As knowledge of biomarkers including circulating nucleic acids markers becomes more refined, many other examples of this pre-diagnosis are likely to emerge. The importance of early detection and appropriate therapy to outcomes cannot be underestimated.

The explosion of complex biological data on disease states has been a major factor in the development of customized, patient—specific therapeutic strategies. Using data derived from biomarker analysis, companion diagnostic approaches can identify those individual patients most likely to respond positively to specific treatments, or those patients for whom adverse drug effects are most likely. The declining cost of DNA and RNA sequencing enhances the patient specific merits of this type of approach and a wide range of examples of the use of biomarkers in prognostic diagnosis or treatment of diseases are now emerging. CD61 is a biomarker found on the surface of drug resistant tumours, by University of California San Diego School of Medicine researchers [13]. CD61 appears to be associated with enhancing tumour metastasis by enhancing the stem cell-like properties of cancer cells. By studying emergent resistance to tyrosine kinase type inhibitor treatments, an ability for CD61 expressing tumour cells to become resistant to anti-cancer agents has emerged, and may be applicable across a range of tumour types, and potentially explain why tumours become resistant to tyrosine kinase inhibitor therapies after prolonged use. Invasive bladder cancers affect up to 400,000 people globally, being amongst the five most frequent cancers in men and the ten most frequent cancers in women. Using a mouse model, Shin and co-workers [14] have shown that in the majority of instances, whether or not a bladder tumour will become invasive can be predicted by monitoring the turning off of a signalling protein, known as sonic hedgehog (shh). The hedgehog pathway appears to inhibit certain steps necessary for bladder tumours to metastasise. This opens up the possibility of targeting therapies to particular types of bladder tumours, in the knowledge of whether a tumour is likely to become invasive, by following shh expression, or of identifying ways to protect shh expression, thereby preventing the onset of abnormal regulation leading to an invasive state [15]. Whist most disease biomarkers to date have relied upon endogenous molecules that can be tracked *in vitro* or *in vivo*, the use of synthetic exogenous biomarkers may provide a means to monitor multiple aspects of disease at the same time. Engineered synthetic biomarkers, combining mass-encoded peptides coupled to nanoparticles have been used to allow non-invasive monitoring of urinary disease traits in a mouse model. The protease-sensitive nanoparticles have been shown to permit disease sites to be identified, allow for sampling of abnormally regulated protease activities and different mass-encoded reporters to be emitted into urine, allowing for multiplex-type detection using mass spectrometry, without the need for invasive biopsies in liver fibrosis and cancer studies. Multiplexing of urinary monitoring using synthetic biomarkers may be useful for a wide variety of disease pathophysiologies and diagnostic systems, which could be expanded to include lipases, nucleases or glycosidases in the future [16].

Whilst tumours remain a major cause of death especially for lung and bladder carcinoma in which it is not easy to make a diagnosis during the initial stages, as yet there are no simple tests which may be applied to large populations. Most current diagnostic methods are complicated, expensive and invasive such as spiral tomography or cystoscopy. For many years attempts have been made to find some markers in blood and other biological fluids, but these have generally not been specific or not sufficiently sensitive for use. Analyses of DNA mutation, of DNA methylation, or of histone modification state have not yet yielded any conclusions especially for the most important tumours like pancreas, lung and bladder. During the last two years particular attention has been placed on the proteomic analysis of proteins and on miRNA.

The presence of a specific DNA mutation does not indicate that a tumour is present or will necessarily develop, as some environmental or epigenetic factors may be needed for expression of the mutation and development of a tumour. The analysis of proteins may give more precise indications. Proteomic analysis of plasma proteins has been undertaken in many tumours , but the large amounts of proteins present creates difficulties in precise identification and quantification of specific proteins. Generally samples from patients affected by tumours, such as non-small cell lung, were pooled and compared with healthy controls to assess protein differences. Subsequent analyses quantified changes in expression of these different proteins. This established that the mechanism of the treatment of lung tumours with citreoviridin, an inhibitor of ectopic ATP synthase, was due to proteins involved in glycogenesis which increases in lung tumours and whose inhibition reduces their proliferation activity [17]. Such analysis was also applied to pancreatic carcinoma [18] and to uveal melanoma [19] when cells were isolated from the tumour and from two metastases of the same tumour. Cells were cultivated and proteins secreted into the medium were analysed. A significant difference was observed with the protein HSP27 which was present in the metastases but not in the original tumour [19]. This protein may have a role in stabilising the structure of cells which may be important for the process of metastatisation.

Another analytical test given strong consideration in recent years is the analysis of miRNA. These RNAs are a single helix of 19-25 nucleotides, synthesised from sequences which are not translated in the gene and modify mRNA by linking to complementary sequences and destroying them. miRNA is stable and resistant to degradation during the process of fixation and histological treatment and for this reason it is considered important as a possible source for diagnosis from preserved tumour samples. Whilst it is not clear if miRNAs are secreted from cells or simply released, or how they are protected from degradation, the more accepted hypothesis is that miRNAs are protected by links with one protein of the group Ago 2.

miRNAs are present in all biological fluids and for this reason and their degradation resistance are considered particularly useful as specific markers. Analysis of specific miRNA has recently been undertaken on lung tumour and specific differences were indicated by comparing the miRNAs in healthy control and tumour affected patients. The expression profiles of miRNAs in mice and human lung are very similar, indicating evolutionary conservation of miRNA expression. It has been reported that lung is one of the tissues with the most abundant expression of miRNA let-7. Besides regulating the expression of known oncogenes and tumour suppressors, miRNAs also act as oncogenes and tumour suppressors directly, providing an apparent connection between the altered expression of miRNAs and cancer development. Thus, miRNA-expression profiles of human tumours is closely associated with diagnosis, staging, progression, prognosis and response to treatment. Differentially expressed miR-29, miR-99b, miR-102, let-7-2and let-7f-1 have all been used to

discriminate histological types of lung carcinomas [20]. Whilst it may not yet be possible to conclude that these miRNAs are derived from lung tumour cells or from the environment, considering their resistance to degradation over time, they are very likely to be protected by microvesicles or lipids. This method shows promising signs of allowing lung tumour diagnosis two years before that made with common methods [20].

Recently a simple RTPCR method permitted evaluation of a large number of miRNAs present in the blood [21]. By analysing 100 miRNAs present in the plasma of healthy and tumour affected patients they were able to distinguish between the miRNA derived from blood cells and therefore present after haemolysis from others which are more resistant and are not degraded, after up to two years in samples stored at -80 °C. Twenty four miRNA were selected and compared by ratio between diseased and control samples. Levels of mRNA 660 and 142-3p declined in blood cancer patients, whereas an increase in miRNA 197 was observed.

4. CRISPR-Cas and genome editing

Clustered Regularly Interspersed Short Palindromic-Repeats (CRISPR) genome editing allows for precision genetic modifications, and when used with suitable guide and tracer RNAs, permits unique sites of modifications to be designed at the whole genome level, as well as at the more conventional genetic element level. The power and universal utility of this tool, and indeed others such as TALENS, have been likened to the restriction endonuclease and PCR revolutions of previous decades. CRISPR tools are frequently combined with the use of the programmable DNA endonuclease Cas9, and have already been applied to more than 25 species, from viruses to rice and from zebrafish to monkeys [22, 23]. The rate of CRISPR-Cas applications development is accelerating dramatically. This approach to precision targeted genome modifications is speeding up breeding cycles in mice from 6 months to 3 weeks and has a vast range of potential applications from gene silencing to whole genome wide functional screening techniques and visualising the constantly changing dynamics of genomes. CRISPR-Cas will prove especially important for disease modelling studies, has recently successfully been used by Chinese scientists in microinjected cynomolgus monkeys (*Macaca fascicularis*) to engineer primates with specific target mutations. Specific mutations in the Ppar-y gene, influencing metabolic regulation and in the Rag1 gene required for immune system health have so far been investigated [24]. Whilst this demonstration of precisely targeted modifications has not yet led to practical applications in biomedicine, establishing the ability to do so in a primate model will prove invaluable to investigating disease states where 2,000 mutations appear to be associated with 300 conditions, developmental biology and epigenetic control of gene expression [25].

5. Gene therapy reaching fruition

Hundreds of gene therapy trials have been carried out, many involving the use of adeno-associated viruses as a delivery mechanism. Vision systems provide a fruitful area for gene therapy, since there are a number of good developmental models, ranging from zebrafish to mice, and the effects of any performance changes can be relatively easily measured. Gene therapy has been successfully used to sustainably deliver improved vision , over 6 months, in 6/6 patients suffering from choroidemia, an X-linked progressive form of blindness, due to loss of light harvesting cell function at the rear of the eye [26]. Using an adeno-associated virus delivery system, patients were administered with the CHM gene, encoding the Rab escort protein (Rep1). This approach of using adeno-associated viral delivery methods for the correction of visual system defects may prove a very promising tool for addressing macular degeneration, the major cause of blindness in developing countries in the years ahead, and has already been shown to rescue retinal degeneration with the lysophosphatidylcholine acyltransferase 1 gene in mice [27]. Emerging applications for this approach extend far more widely, including the prospect of organellar gene therapy, using proteins targeted to mitochondria for example, as well as having the potential to correct heart muscle defects and to develop novel gene delivery tools to particular organs, such as electroporation to introduce brain derived neurotrophic factor genes to enhance bionic ear cochlear implant performance in guinea pigs [28]. The power of stem cells to overcome loss of function, for example associated with progressive blindness [29], or in the treatment of multiple sclerosis, where promising results have recently been obtained using human stem cells in mice, to overcome paralysis and a range of other nervous system communication defects [30], should not be underestimated.

6. 3D printing and additive technologies

3D printing is a 30 year old technology which is now becoming increasingly accessible to all, with entry level equipment costs falling to as little as $600 and more than 100 different substrates now having been used to develop prototypes or products. The universal availability of computer aided design tools and worldwide sharing of open access design elements is delivering a creativity explosion combining elements of design, composition, strength and finishing properties to produce a burgeoning number of prototypes and products combining the best of engineering and biomaterials, which can be produced by anyone, anywhere. 3D-printing will deliver massive advances in the screening of therapeutic drug candidates, for example, through the use of cell coated nanospheres, as well as tissue level surrogates, significantly reducing one of the major bottlenecks in product development pipelines to a biotechnology sector with a research and development budget of >$148 Bn annually [31]. 3D printing technologies, and the associated use of engineered biomaterials are already starting to make a difference at a range of levels. Model systems, hoping to break into the $36 Bn organ replacement market, such as Organovo's functional liver slices able to detoxify solutions for at least 40 days [32], and *in vitro* growth of spare ear lobes and bionic ear systems able to deliver stereophonic audio reception [33] are pointing the way for future applications. In clinical settings, 3D printing and engineered biomaterials such as reconstructed pelvic bones [34] and printed titanium lower jaws are, subject to issues of compatibility and potential rejection, proving invaluable to patients of all ages. The ability to rapidly develop new prototypes, for example of how the human foot functions from a biomechanical perspective, as carried out at Glasgow Caledonian University, is proving invaluable to the $18 Bn prosthetics industry and to rehabilitation science. Further benefits from the use of 3D printing and bioengineered materials are likely to include reductions in the use of animals for product testing [35], as more sophisticated printed tissue and ultimately organ models become available. The potential for 3D printed and engineered biomaterials to impact on clinical

practice is shown by the continued successful use of artificial tracheas, over more than five years in patients [36] and of synthetic corneal materials applicable to more than 10 million corneal blindness sufferers globally.

Furthermore, the potential for 3D printed and engineered biomaterials to impact on agricultural practice exists. This is exemplified by studies on and modelling of soil microcosms to understand soil architecture and to inform soil fertility [37, 38]. Using X-ray Computed Tomography (CT) and 3D printing significant insights into the complex pore geometry of undisturbed soils can be made. Since it is difficult to study and map real soil and to control or manipulate it, artificial soil and models are possible and quite valuable. The effect of soil architecture on fungal invasion as a consequence of pore geometries can be used to parameterise a model of fungal growth and interactions. Model structure can be augmented with carbon distributions to disentangle the effects of structure and carbon which is difficult to do in real soil systems. These 3D printed microcosms are reproducible; enabling replicated heterogeneous structures for experimentation that reflect well with those of real soils. The fungal growth model can identify which traits promote colonisation in different environmental contexts and permits simulating and observing interaction outcomes between fungal pairs and mapping fungal interactions amongst a community of intrinsically different individuals [37-39]. It is also feasible that knowledge of this fungal interaction network and organisation with soil microcosms can be extended to understanding cancer invasion and metastasis.

7. Bionanomaterials

The nanotechnology revolution, when allied to insights from stratified and personalised medicine, points the way towards dramatic improvements in the targeted delivery, controlled dose and release rates of novel therapeutics. Nanoparticle biosynthesis is a highly flexible and accommodating system for the production of novel therapeutics or biotechnological tools [40]. More than 20 nanoparticle therapeutics have now received clinical use US Food & Drug Administration approval [41]. Doxil, consisting of the therapeutic deoxorubicin wrapped in not one, but two liposome derived coatings, is an early example of how this approach can be used to maximise delivery to target tissues, increasing the potential efficacy of treatments, and improving outcomes for Karposi's sarcoma, ovarian cancer and myeloma patients, although some polymer toxicity issues require further monitoring. Even more promising for the future are combination smart bionanomaterials, which will combine modules with different functions, so that particular target cells are identified by signal modules, recognized and bound to by separate effector modules, giving greater degrees of accuracy in treatment [42]. Future prospects for bionanomaterials will also include the use of graphene family nanomaterials, perhaps only a single molecule in thickness, of a variety of shapes and functions, not just limited to biomedicine[43], but with important applications in biosensing, gene delivery, advanced tissue engineering [44] and real time imaging [45]. Advances in synthetic biology will also influence bionanomaterials properties, uses and production as more imaginative and practical applications combining elements of biological networks and nanoscience enables new materials to be created [46, 47]. Flexibility and potential for imaginative new products to be created is demonstrated by advances in origami DNA, whereby long DNA strands can be shaped into almost any three dimensional nanostructure [48]. This has applications in biocomputing, using DNA based robots to act as logic gates, channels and components of artificial machines which has recently been extended to living cockroaches (*Blaberus discoidalis*) to control cell targeting [49].

8. Wearable devices

Point of view information during operations for image analysis, combined with voice commands and involving external collaborators in surgery offers powerful tools to increase effectiveness and value as an educational resource. This approach has been successfully used at Ohio State University, for example, in operating to repair knee cruciate ligament, and in gastrostomy [50]. The enhanced sensitivity of Google Glass derived apps and server platforms for removing subjectivity from high throughput screening of rapid tests demonstrates the utility of wearable devices, as recently shown at the University of California Los Angeles, to revolutionise diagnostics [51].

The potential for wearable devices to be used in routine telehealth is also shown by recent plans for Apple to expand into the health sector, described by CEO Tim Cook as 'primed to explode' [52]. The much-rumoured iWatch, may for example, have blood-sugar level and nutrition monitoring functions built in, as well as tracking activity undertaken, and remotely send on a range of other physiological functions such as blood pressure, heart rate, temperature, hydration and anti-oxidant status. Apple are believed to have assembled high powered teams of biomedical scientists, engineers, software and hardware gurus from a range of telehealth and sensor technology companies, including Vital Connect, Sano intelligence and O2 MedTech, further telehealth wearable device developments are inevitable.

9. Citizen science

Novel applications of technology are not just limited to industrial or research led processes. Individual citizens can become involved in the testing or analysis of biotechnological data through 'Citizen Science' initiatives. Whilst mobile phone apps enabling the public to record and report incidences of unusual plant diseases, which can be identified and mapped remotely, citizens can also become directly involved in the analysis of original data generated through large, interdisciplinary projects. Such initiatives frequently involve members of the public in the analysis of genomic or microarray data, to assist in mapping or three dimensional modelling projects. The ubiquity of powerful computing tools in our everyday lives allows us all to contribute as Citizen Scientists to the advancement of knowledge- and have some fun in the process. Although perhaps not yet as advanced as the latest Nintendo Wii fitness applications, we can now participate in bioinformatic analysis of fungal pathogen genomes and host resistance genes using apps such as the Fraxinus Facebook game (http://apps.facebook.com/fraxinusgame/), which uses genetic data from the *Chalara fraxinea* fungus causing ash dieback throughout Europe and North America and gets the player to spot genomic DNA patterns using rows of coloured leaf shapes. The complex nature of ash dieback transmission, involving asymptomatic hosts, global markets for plants and incomplete knowledge of how fungal transmission might be limited has meant that ash dieback is currently out of control in many countries. Cancer genomic data can be analysed using a space race game app, such as Cancer Research UK and

Guerilla Tea's 'Genes into Space', where the journey through space is in reality a journey through peoples' chromosomes, looking at gene copy numbers and mapping where breaks occur[53]. This amusing pastime enables any citizen to contribute to the fight against the world's greatest killer.

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