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Toward understanding and exploiting tumor heterogeneity

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The extent of tumor heterogeneity is an emerging theme that researchers are only beginning to understand. How genetic and epigenetic heterogeneity affects tumor evolution and clinical progression is unknown. The precise nature of the environmental factors that influence this heterogeneity is also yet to be characterized. *Nature Medicine*, *Nature Biotechnology* and the Volkswagen Foundation organized a meeting focused on identifying the obstacles that need to be overcome to advance translational research in and tumor heterogeneity. Once these key questions were established, the attendees devised potential solutions. Their ideas are presented here.

In many malignancies, molecular and cellular heterogeneity within a single tumor, between different sites of neoplasia in a single patient and among tumors from different patients confounds researchers' understanding of tumor evolution and their ability to design and select effective therapies and curtail treatment resistance[1–3].

Researchers are still, however, at the very beginning of understanding the full extent of tumor heterogeneity (including the contribution of the tumor microenvironment), which types and aspects of tumor heterogeneity are relevant in which tumor types and in which clinical scenarios, and how to counter and/or exploit tumor heterogeneity for therapeutic gain.

To begin to tackle these issues, *Nature Medicine*, *Nature Biotechnology* and the Volkswagen Foundation invited 20 scientists from around the globe for a two-day brainstorming session in the beautifully restored Herrenhausen Palace in Hannover, Germany (**Fig. 1**). Reflecting the variety of expertise needed to tackle the issues mentioned above, this group included computational biologists, technology developers, cancer biologists, clinicians, industry representatives and regulators. The aims were to identify the most important questions about tumor heterogeneity and map paths to answering them. We hope the new collaborations and networks forged at the meeting will help make some of these paths a reality.

All in attendance felt that sharing the group's findings—especially the questions identified as most pivotal—with the broader community was key. This Perspective aims to do just that and is organized in the same manner as the meeting. Whereas the first day involved all attendees brainstorming as a single group about the most important questions, the second day saw four smaller discussion groups ('cancer evolution', 'beyond the genome', 'clinical and regulatory' and 'technology') brainstorming about the answers to four or five select questions. At the end of the second day, each group presented their conclusions to the larger group. The question-and-answer period that resulted proved to be a highlight of the meeting.

Cancer evolution

Many biological aspects of tumor heterogeneity are unknown, but the group focused on establishing the basic premises by which we can define and study the parameters of tumor evolution.

What is a clone? The term 'clone' is used widely in the field, but discussion in this group revealed that, perhaps surprisingly, there is no consensus about what it indicates; in fact, this question sparked some of the most animated discussion at the meeting. In principle, under the assumption that tumors arise from a single cell, each tumor can be considered a clone. In this scheme, trunk mutations—also called founder mutations—that are present in every cell have a cancer cell fraction (CCF) of 1. All cells within a tumor with a CCF < 1 can be considered subclones, at least in terms of their relative population frequency within a given lesion. However, the group recognized that even this definition is misleading owing to an illusion of clonality within a single biopsy, where a particular mutation can appear clonal in one biopsy, with a CCF of 1, but subclonal or absent altogether in subsequent tumor sampling (**Fig. 2**).

What is a driver? The term 'driver' typically denotes a genetic event associated with tumor initiation or progression. Although it might traditionally be viewed as a tumor cell–autonomous alteration that promotes tumor proliferation, after discussion we felt it would be useful to extend the definition to encompass more of the complex biology of pro-tumorigenic events. In other words, a broader biological definition of 'cancer driver' would be a cell-autonomous or non–cell-autonomous alteration that contributes to tumor evolution at any stage—including initiation, progression, metastasis and resistance to therapy—by promoting a variety of functions including proliferation, survival, invasion, or immune evasion. Notably, such an alteration could be the result of direct mutational events, including

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Figure 1 Herrenhausen Palace. Image credit: Eberhard Franke for Volkswagen Foundation.

genetic and epigenetic events, or of aberrant signals and mutations in one or more of the driver's regulators and cognate binding partners. Accordingly, candidate drivers can be identified through a variety of methodologies, including statistical analysis of genetic or epigenetic alterations, functional screens and analysis of regulatory networks[4]. As such, they must be confirmed by experimental evidence, including preclinical *in vitro* and *in vivo* data and clinical data. Complicating the matter further, as the role of a driver is constrained by spatial and temporal contexts, genetic events can act as drivers at one stage of tumorigenesis and as passengers at another stage, and vice versa.

What is the source of heterogeneity in cancer, and what is the contribution of heterogeneity to cancer evolution? Heterogeneity in cancer is driven by two principle factors: the introduction of genetic (or epigenetic) alterations mediated, for example, by genomic instability, and the evolutionary selection thereof. Notably, although evolution is driven by selection of phenotypes according to their relative fitness, not all somatic genetic alterations have a recognizable phenotypic consequence, and even fewer provide a fitness advantage. Selection for phenotypic alterations can favor the outgrowth of cells with genetic alterations associated with that phenotype. Therefore, in the study of cancer evolution, it is likely that functional screening combined with multidimensional phenotyping—measuring signaling, epigenetic, transcriptional, metabolic and other alterations in addition to genetic alterations—will be most informative in revealing the sources of the phenotypes driving tumorigenesis. Generating and interpreting these data is not trivial, and the unanswered technological questions related to these issues are covered below. Regarding the contribution of heterogeneity, although heterogeneity can be broadly considered to be a trait that allows tumors to overcome evolutionary pressures, it can also reflect vulnerabilities that could be exploited therapeutically. This makes it even more important to develop tools to quantify and model tumor heterogeneity.

How can tumor heterogeneity be modeled in preclinical experiments? One challenge in assessing the dynamic contribution of heterogeneity as a trait of tumor progression is the fact that current preclinical tumor models do not recapitulate the condition under which heterogeneous tumors arise and evolve in humans[5]. For example, although genetically engineered mouse models (GEMMs) have been instrumental in revealing crucial aspects of tumor biology, tumors in these animals need to be analyzed when they are relatively small, for ethical reasons. The tumors are also homogeneous, driven by a small number of genetic alterations, and can be polyclonal in nature, in contrast to the monoclonal nature of the majority of human cancers. Tumor burden, metastatic potential and tumor longevity are also not recapitulated adequately in mouse models. New technologies need to be applied to these problems. For example, clustered regularly interspersed short palindromic repeat (CRISPR)–CRISPR-associated protein 9 (Cas9) genome editing, perhaps used in combination with existing GEMMs designed by transgenic or viral expression of tumor-driving alterations, can help recapitulate the genetic complexity accumulated during human tumor evolution. Patient-derived xenograft (PDX) models capture, at least initially, some of the heterogeneity of patient samples. However, subclones can be selected for increased fitness for growth in the mouse host, which lacks the proper microenvironmental and immune components that may otherwise influence subclonal selection. Ongoing efforts to humanize mouse models may help incorporate relevant features that shape tumor evolution in humans, but differences in the longevity and size of the mouse compared to the human, together with ethical considerations inherent in the conduct of mouse experiments, will probably limit application of these models to the human disease. Beyond animal models, *in vitro* approaches such as tumor slice cultures can be exploited to recapitulate a snapshot of the tumor in its native environment, and organoids can be used to model tumorigenesis in human cells. *In silico* models that use multiscale parameters can also create interesting hypotheses that are experimentally testable. However, because no model is perfect, many in the group felt that there was no substitute for studying tumor evolution in patients.

Beyond the genome

What is the contribution of the epigenome to tumor phenotype and clinical outcome? Cell states are defined by the interplay of the genome, epigenome, transcriptome and proteome in each tumor cell (**Fig. 3**). Because cell states tend to be self-stabilizing, there are typically fewer distinct cell states in a tumor than the degree of genetic, epigenetic and transcriptional heterogeneity would suggest. Thus, even genetically distinct cells may be in a similar cell 'state' and hence may be susceptible to treatment with the same drugs. On the other hand, even genetically identical cells can exist in different cell states, owing to epigenetic differences and influence of the microenvironment. But it is time to stop thinking about the genetic and epigenetic contributions to cell state separately, because their contributions to cell state may be intertwined. Furthermore, epigenetic defects, such as promoter CpG island hypermethylation–associated silencing of DNA repair genes, are known to cause genetic changes, and translocations and mutations can cause epigenetic disruption, which creates mutual dependencies between epigenetic and genetic traits.

Researchers must strive to identify relevant cell states in cancer by integrating different data sets and, once these are identified, work toward therapeutic strategies based on inferred cell states. Epigenetic data form only a part of such integrative analysis, but epigenetic modifications are dynamic and responsive to environmental pressures, so they may exert a particularly strong role in the definition of the cell state and behavior at any given moment in response to therapy. In addition, although epigenetic marks are dynamic, they represent the history of the cancer: once a cell has passed through a particular cell state, some of these epigenetic marks remain. Moreover, epigenetic marks can also reflect the potential of the tumor to respond to an environmental or therapeutic pressure. Epigenetic marks are therefore unique in their ability to provide information about the previous, present and potential future states of a cell. They can also provide a built-in 'barcode' that can measure a tumor's epigenetic clonality[6].

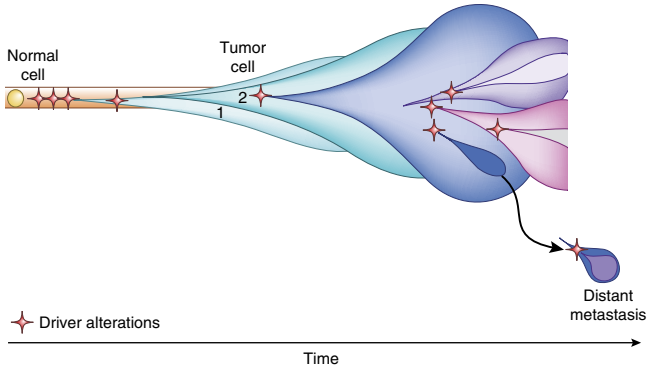

Figure 2 The clonality of tumor evolution. In tumor evolution, driver alterations may result in the formation of the initial tumor clone. As further driver alterations occur, these clones then branch off to form subclones. Founder mutations that occur in the original tumor clone are hard to identify, however, as a mutation at point (1) may be considered to be a founder mutation, but further mutations at point (2) would have been considered to be in the initial clone had the sample at point 1 not been taken.

Because epigenetics provides a different and complementary paradigm to the analysis of genetic mutations, it may be possible, once these states have been defined, to use two or three important epigenetic markers to infer cell states. Furthermore, as the epigenetic state of cancers is more plastic than that of normal development, such contributions may be critical to understanding phenotypic changes of cancers, such as the epithelial-mesenchymal transition, the capacity to disseminate beyond the primary site and drug resistance.

What methods and samples are needed to describe and understand the heterogeneity and influence of the tumor microenvironment? To understand the influence of the microenvironment on cell state, researchers need to coordinately characterize DNA sequence, epigenome, transcriptome, protein, metabolites and infiltrating immune cells in both the tumor and the stroma. Evaluation of data from single cells will provide additional insight into heterogeneity. Only through integration of such data, using either statistical and machine learning approaches or analysis of regulatory and signaling models, can we begin to develop a more robust understanding of cancer states. As a consequence, there will continue to be increasing need for computational biologists. Such technologies are discussed further below.

How can the immunogenicity of tumors be increased? Immunogenicity depends, in part, on mutations that generate epitopes that are not recognized as self by tumor-infiltrating T lymphocytes. Therefore, chemotherapy and other genotoxic drugs may improve the outcome of immunotherapy interventions, including adoptive T cell transfer and immune checkpoint blockade, by generating mutations or modifying the immune microenvironment[7]. However, it is unclear whether subclonal changes in immunogenicity are enough to cause the whole tumor to be eradicated by the immune system. It is possible that applying radiation therapy before checkpoint blockade will result in increased efficacy. Isolated cases have suggested an abscopal effect of such treatment, but this has yet to be confirmed in a randomized clinical trial[8]. Recent data suggest that the sustained benefit of radiation combined with blockade of cytotoxic T lymphocyte–associated protein 4 (CTLA4) may also require blockade of programmed death-ligand 1 (PD-L1) to reverse T cell exhaustion and that radiation increases the diversity of the T cell–receptor repertoire on intratumoral T cells[9]. Oncolytivc viruses may also be used to increase immunogenicity via the induction of an inflammatory response upon local injection of virus, leading to control of distant tumors by an increase in tumor-infiltrating cytotoxic populations[10]. For example, Talimogene laherparepvec (T-VEC) has shown promising data in phase III clinical trials[11].

Clinical and regulatory considerations

What aspects of tumor heterogeneity matter in the clinic, and how can they be transformed into diagnostic strategies and treatment guidelines including biomarkers of response? There is a dearth of information on the degree to which heterogeneity affects the clinical management of patients. More work to document the phylogeny and generate atlases or road maps for each tumor subtype is needed. This will enable more confident identification of trunk mutations for each subtype and understanding of the branching properties. One could easily assume that between the Cancer Genome Atlas (TCGA), the International Cancer Genome Consortium (ICGC) and other consortia the tumor genomic data needed to generate road maps for each tumor subtype are readily available, but the discussion group felt that none of the existing tumor genome repositories are sufficient for this sort of analysis. This is because these programs were not designed to address the heterogeneity component of cancer, and they use platforms that characterize tumors in 'bulk', giving results that average across all tumor clones. Although bioinformatics tools have been developed to tease out the clonal data in these data sets, these inherent limitations still exist. What is needed for each subtype is a minimum number of primary tumors; the minimum number is likely to vary according to the tumor subtype and its inter-patient heterogeneity. Ideally, researchers would obtain multiple regions from each tumor to capture spatial heterogeneity. To differentiate trunk mutations from subclones, each region must be sequenced deeply. Epigenetic and other analyses should also be performed. Patients who donate tumors must then be followed longitudinally, and their tissue—where practical—and blood should be collected at regular time points and subjected to deep sequencing to track the molecular changes. Clinical annotation of samples and phenotypic correlation is essential at each step. This sort of analysis should reveal a finite number of trunk (clonal) and tree (subclonal) mutations, which can inform on the signaling pathways involved, for each tumor type. Excitingly, some new studies, such as TRACERx (Tracking Non-small Cell Lung Cancer Evolution through Therapy (Rx); NCT01888601), incorporate several of these design elements, albeit in a single tumor type.

How can we maximize the extraction of molecular and clinical data that are sharable and likely to lead to benefits for patients? The research community needs new consortia composed of academic medical centers, industry partners and regulatory agencies. Prior to clinical sample collection or data generation, all stakeholders need to agree on a minimal set of metadata that need to be collected for each tumor in a format that enables sharing; characterization must be systematic and agnostic to the tumor subtype. Genomic, clinical and any other data—once collected—must be added to a suitable repository and within an agreed-upon time frame. To maximize the extent of effective data sharing and minimize limitations caused by differences in consent practices across institutes, municipalities and nations, new harmonized consent practices consisting of either universal consent forms or an option for patients to waive all restrictions on global sharing of data—even data, such as germline genetic variants, that have the potential to reveal the identities of patients and their relatives—are needed up front. Patients should be empowered to drive data

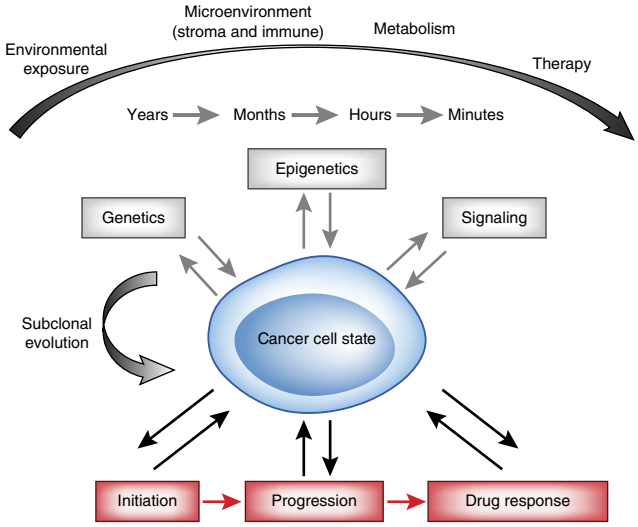

Figure 3 Influences on cancer cell state. The cancer cell state is representative of the genetic and epigenetic components as well as the signaling environment, which collectively determine which genes are expressed by the cell. These properties may be altered by subclonal evolution and can influence the initiation, progression and drug response of the tumor, affecting both the bulk tumor and the single-cell state. The gray arrows show the timescales of these processes and the factors that might influence cell state on each timescale are indicated.

sharing—through formation of new patient advocacy organizations, for example. Encouragingly, several of these considerations are being incorporated into new consortia such as Cancer Core Europe[12].

How can tumor heterogeneity be 'drugged'? Although combination drug studies are challenging, adaptive trial designs to test combinations of targeted therapies with chemotherapies and/or immunotherapies based on molecular information extracted from individual tumors will be needed. Whether these combinations are given simultaneously, at the start of treatment, or sequentially, as new resistance or other subclonal mutations appear during longitudinal analysis of patient samples obtained through noninvasive methods, may vary depending on the road map of each tumor subtype and on the therapeutic window of each drug alone and in combination. Ideally, we could always target druggable trunk mutations and then add drugs to block emerging subclones. To simplify the development of combination therapies, drugs showing a high degree of tumor selectivity (for example, those targeting a mutant but not wild-type version of a tyrosine kinase) may be prioritized. To minimize legal and financial hurdles that prevent testing of combinations of different drugs from different companies, 'honest broker' approaches that negotiate these issues with companies (along the lines of the Cancer Research Institute Clinical Accelerator, Cancer Core Europe and the US National Cancer Institute Cancer Therapy Evaluation Program) should be proactively incorporated into the consortia mentioned above. It is likely that tumor heterogeneity in the form of increased somatic mutational diversity represents, in some cases, an Achilles' heel for tumors owing to the increased likelihood of tumor neoantigens being recognized as non-self by T cells.

In addition, we need to increase researchers' ability to dissect the specific molecular mechanisms that contribute to drug synergy and complementarity in combination therapy. For instance, a recent project in the DREAM Challenge, an open science effort by a nonprofit community including researchers from academic institutions and companies, collected analyses from 31 labs around the world on the prediction of drug synergy in human lymphoma[13]. The study revealed that, despite a complete lack of prior literature on drug synergy prediction, several labs can now effectively predict synergistic combinations that are experimentally validated. Yet most of the approaches are still relatively naïve and will benefit from a more systematic and concerted effort to characterize drug mechanisms of action and activity at the molecular level through predictive and computational approaches.

Why do clinical trials fail, and what is the clinical trial of the future? The ideal clinical trial will incorporate patients whose tumors have been selected as likely to respond on the basis of molecular markers that have been well validated in preclinical studies. However, trials in which a single agent is tested in a cohort with a matched biomarker do not provide information about the impact of heterogeneity or the longitudinal evolution of clonal or subclonal cells. The reason for lack of response, in a cohort or at the individual level, requires understanding of the spatial and longitudinal heterogeneity of the tumor. The ideal clinical trial will respond—in real time—to molecular changes, revealed by frequent characterization of tumor evolution, in response to therapy. This characterization will require material from the primary tumor or metastases (not always accessible) or could be achieved by studying nucleic acids or cells in the blood, as emerging data suggest liquid biopsies are feasible[14–17]. Imaging approaches may not have sufficient resolution, information content or speed to reveal molecular changes indicative of emerging resistance to therapy, although new imaging modalities such as ^{13}C-based magnetic resonance spectroscopy might provide metabolic readouts of response[18]. Broad changes in clinical practice and regulatory procedures may be needed. For example, are we ready to conduct trials in which treatment is adapted on the basis of changes in circulating tumor DNA as an indicator of progression? Similarly, if a resistance mutation is detected in a patient's circulating tumor DNA but imaging analysis shows that the tumor is stable or shrinking, would a clinician be comfortable switching to a different targeted therapy? Although changes to include targeted therapies have been adopted in the treatment of some malignancies, including chronic myeloid leukemia, broader changes may be needed.

Technology

Which sources of heterogeneity can be measured, and which are difficult to assess with regard to DNA? High-throughput DNA sequencing of bulk samples is the most common of all the technologies used for the molecular characterization of tumor heterogeneity. Single-nucleotide and structural variations with a high allele frequency can be robustly detected at the sequencing depth routinely achieved in experimental and clinical settings. For comprehensive cataloging of mutations that occur with a frequency of less than 1–2%, the sequencing depth required for robust variant calling (400–500×) is still prohibitive for larger-scale studies, but with the continuing development in sequencing technology, this issue is likely to be solved in the near future. A major advantage of DNA sequencing is that it is relatively robust to sample treatment, and high-quality data can be obtained from most specimens, although accurate enumeration of subclonal tumor heterogeneity in formalin-fixed and paraffin-embedded (FFPE) archival samples can be challenging.

In the context of heterogeneity, the more recent development of single-cell genome sequencing is very exciting, as it enables not only estimation of the frequency of individual mutant alleles in a cancer sample but also determination of co-occurring or mutually exclusive

alterations. By sequencing and comparing multiple single cells, it is possible to reconstruct cell lineages and phylogenies using mutations as stable markers of evolution. Single-cell genome sequencing also enables study of intratumor heterogeneity in rare subpopulations such as circulating or disseminated tumor cells. Currently, the main limitations for single-cell genome sequencing are its relatively low throughput and its high cost, partial genome coverage and uniformity, in addition to technical problems, such as allelic-dropout errors and false positives introduced by whole-genome amplification methods. The throughput and cost issues can be addressed by performing genome sequencing on targeted regions such as the exome or cancer gene panels in single cells. Algorithms for calling single-nucleotide variants, insertions and deletions, copy-number profiles and structural variations have not yet been developed for single-cell data, but they are desperately needed owing to the inherent differences in the data.

Regarding DNA. As with DNA, sequencing is now the method of choice for investigating the RNA composition of tumors. In contrast to DNA data, it is difficult to learn much about the heterogeneity of bulk samples from RNA sequencing (RNA-seq) data, beyond what can be gathered by sequencing samples from different regions of the tumor. The tumor microenvironment may represent as much as 90% of some tumor samples and contributes proportionally to the RNA pool, which affects measures of heterogeneity and the resulting transcriptional profile. Computational deconvolution of different expression components in a sample can distinguish between cells from different lineages but have limited applicability in samples with low transcriptional diversity[19,20].

Single-cell RNA-seq is a robust technology[21] that, with the emerging Drop-seq, in which individual cells are separated into nanoliter-sized aqueous droplets and sequenced, and microwell sequencing[22–24] methods, can analyze tens of thousands of cells simultaneously in a cost-effective and efficient manner. That said, sensitivity for lowly expressed genes still needs to be increased for all RNA-seq protocols and better methods for controlling amplification biases and technical noise are needed. Optimized analytic tools for single-cell RNA-seq methods are also being developed, but a thorough comparative benchmarking of these tools is needed. For single-cell methods, obtaining full-length RNA sequences or information about RNA modifications remains challenging. Furthermore, the current throughput of next-generation sequencing platforms is insufficient to profile tens of thousands of single cells.

As the transcriptome is highly dynamic, sample handling is a critical hurdle in the acquisition of quality transcriptomes. Issues to consider include how quickly the sample is processed or frozen after its extraction from the patient and, even more importantly, the protocol by which the cells are disassociated from solid tumors. The availability of fresh or rapidly frozen samples is essential; FPPE samples can be processed for RNA-seq but are unlikely to provide a reasonable picture of cancer cell states, and their often fragmented nature will preclude discerning completely phased, or linked, genetic information.

Optimizing analysis pipelines for variant calling has been an intensive focus of research in recent years. A low false-positive or false-negative mutation detection rate has little effect in cohort studies, such as those by the TCGA or ICGC, but may lead to artifactual differences between related mutation profiles and cause critical misinterpretations of study results. What is needed is an independent systematic evaluation of the many pipelines currently used for mutation calling in cancer samples, as has been started for the baseline 'normal' genome with the Genomes in a Bottle Consortium. A valuable community resource would be the availability of benchmarking reference specimens with defined clonal composition as assessed by a gold standard. We expect that results from comparative evaluation, such as the ICGC-TCGA DREAM Genomic Mutation Calling Challenge, a consortium set up to improve mutation calling within cancer genome sequencing data, will provide a good estimate of the relative performance of methods for processing whole-genome data sets, but further investigations are likely to be needed to benchmark tools for calling of subclonal mutations and the estimation of allele frequencies. Conservative approaches, such as >60× coverage thresholds and mutation filtering using multiple normal (germline) samples, are recommended when determining the degree of heterogeneity between tumor samples. A relatively uncharted area is the development of metrics that quantify similarity and difference between samples from the same clonal origin, such as multiple biopsies from the same tumor, pre- and post-treatment samples from the same patient or tumor samples and (xenotransplanted) model systems.

Regarding protein. Techniques for investigation of proteins lag behind those for nucleic acid analysis, especially in terms of sensitivity and comprehensiveness. It is possible to get a complete picture of the protein content of a sample using mass spectrometry–based proteomics, but at the moment the relatively large amount of material needed makes proteome-wide experiments on cancer samples unfeasible in most instances. Antibody-body based techniques are the method of choice when sample material is limited, but they are limited by throughput and the availability of high-quality antibodies. At the single-cell level, technologies such as fluorescence-activated cell sorting (FACS) or mass cytometry (CyTOF) allow the investigation of up to about 17 proteins per cell (FACS) or 45 proteins per cell (CyTOF) with very high throughput. Future development of CyTOF technology might increase the number of proteins that can be monitored, but no technology that can provide a truly comprehensive protein atlas for single cells is on the horizon.

Protein content is less dynamic than the transcriptome or the epigenome, which reduces the requirement for sample freshness. However, the phosphoproteome, which is critical for the understanding of cancer signaling, is even more sensitive and rapidly changing than the transcriptome. FFPE samples can be processed for proteomics experiments but not for CyTOF or FACS.

As discussed above in the section 'Beyond the genome', the epigenetic features of chromatin, including histone modification, DNA base modifications (such as methylation and hydroxymethylation) and DNA accessibility, provide information about both the cell state and the evolutionary history of a tumor. Robust technologies have been developed to provide genome-wide maps of most epigenetic marks. For histone marks, some methods of chromatin immunoprecipitation combined with next-generation sequencing (ChIP-seq) can be reliably applied to very small samples (1,000 cells or fewer). Several techniques are routinely used to assess methylation levels. The most comprehensive picture can be obtained from whole-genome bisulfite sequencing (WGBS), but precipitation techniques (methylated-DNA immunoprecipitation sequencing and methylated–DNA-binding domain sequencing) or reduced-representation bisulfite sequencing (RRBS) are also in use. WGBS can be applied to small samples, but the DNA-damaging effects of bisulfite treatment limit genome coverage. The development of alternative chemistries that are less harsh will help reduce experimental artifacts. Illumina's Infinium HumanMethylation450 BeadChip platform provides an array-like alternative that has been found to provide acceptable DNA methylation profiles, even with FFPE samples. Assays for the various oxidized

forms of 5-methylcytosine have been developed but not thoroughly validated in terms of reproducibility and sensitivity. DNA accessibility and nucleosome positioning can also be readily measured, most commonly by DNAase I–based assays and, more recently, by transposase-accessible chromatin using sequencing (ATAC-seq) for bulk and single-cell samples. As there are a large number of epigenetic modifications of interest in any given sample, a big need in the field is the development of multiplexing strategies that allow measurement of many marks at the same time in the same sample, although nascent methods to examine multiple chromatin marks within a single stretch of chromatin exist[25].

Some estimation of cellular heterogeneity can be obtained from bulk experiments in the case of DNA methylation, but single-cell assays, although limited by throughput, would provide advantages in terms of capturing the amount of heterogeneity and determining the degree of co-occurrence and phased states of epialleles. Single-cell assays for histone marks have yet to be developed, but given the rapid development in the field, single-cell ChIP-seq assays can be expected in the near future.

The epigenome, like the transcriptome, is highly dynamic and sensitive to changes in the environment. As such, fresh or rapidly frozen samples are essential for its study. Finally, a wide range of RNA base modifications, collectively called the epitranscriptome, have been implicated in translation control, RNA splicing defects and many cancer types[26]. These dynamic marks are also likely to possess some degree of heterogeneity, although single-cell methods are needed to tease out such varied RNA states within and between cells from within a tumor.

A complete picture of a cell state will often require measurement of different parameters in the same cell. Although it is usually possible to perform multiple assays on a bulk sample, only in some cases is this possible with single-cell measurements[25,27]. Further development of multimodel measurement methods will aid understanding of the relationship between point mutations and gene expression and between methylation changes and changes in the expression of oncogenes, for example.

How can we assess spatial organization of tumors? Traditionally, when a spatial resolution higher than what can be achieved by multiple biopsies is desired, assessment of spatial heterogeneity in tissue samples has been limited to microscopy-based methods. For example, immunofluorescence and fluorescence *in situ* hybridization can localize proteins, RNAs and DNA mutations in tissue slices with high sensitivity, potentially down to the single-molecule level. In practice, both methods suffer from difficulties in quantifying expression levels and in comparing results within and between samples, owing to variable background and target accessibility. These techniques are also very low throughput, and only a handful of mRNAs or proteins can be imaged simultaneously with standard technology. Imaging site-specific epigenetic modifications is currently not routinely done, although at least one method has been developed to visualize histone modifications in fixed tissues[28,29].

Excitingly, recent years have seen the emergence of new technologies that promise to revolutionize our ability to assess the spatial heterogeneity of protein and RNA expression. For proteins, CyTOF has been developed into an imaging tool that can image the localization of up to 32 proteins at present (and potentially up to 100)[30] with subcellular resolution[31]. For RNA, *in situ* sequencing methods[32–34] can provide information about the RNA content of individual cells in fixed tissue. The practical applications of these technologies are still in their infancy, and a thorough benchmarking of reproducibility and sensitivity has yet to be done. Throughput of these new technologies still seems to be severely limited at the moment.

With these new technologies on the rise, the critical bottleneck has become the development of computational methods to analyze data from each technology, integrate information from different technologies and connect these data to prognostic and actionable clinical information.

What noninvasive or minimally invasive technologies can be used to obtain information about tumor heterogeneity? Currently, the blood is the best source of information about the molecular makeup of a cancer that can be obtained without biopsying the tumor itself. Cell-free DNA and circulating tumor cells are especially rich sources of information. Few high-quality studies have been done to assess how well data obtained from these blood-borne biomarkers reflects the tumor itself, although a number of recent studies have highlighted the power of this approach for the noninvasive characterization of tumor heterogeneity in carcinomas of the colon, breast and lung[14–17]. It is also unclear whether primary tumors or metastases contribute more to the pool of circulating cancer material. It seems clear, however, that even if circulating material is found to faithfully reflect the tumor itself, there is still a need for more efficient ways of isolating the cells and nucleic acids from the blood and for data analysis tools that can more faithfully reconstruct the parent tumor.

Although current *in vivo* imaging technologies are unable to provide many insights into intratumor heterogeneity in patients owing to resolution and labeling issues, some work indicates that heterogeneity of radiographic imaging, including positron emission tomography (PET) and magnetic resonance imaging (MRI), is clinically predictive of response[35,36]. Image-guided biopsies may also make an important contribution to the analysis of genome-based intratumoral heterogeneity by providing the spatial context to relate different regions.

How should methods for assessing heterogeneity be benchmarked and validated? Validation of the accuracy and robustness of the assays discussed above will require the development of gold-standard samples that are readily available and can be recreated in reproducible manner by individual labs. For some data types, such as DNA mutations, simple mixtures of cell lines will be sufficient, but others, such as epigenomic or RNA expression data, are too sensitive to environmental changes and will require test samples with more intrinsic control of biological variation, such as 'spike-in' standards. For each assay, a set of quality-control metrics that can be used to assess the performance of improved methods and that investigators can apply to their own experiments will have to be agreed upon.

One should also keep in mind that the degrees of accuracy needed for understanding biology and for informing clinical decision-making may differ and should be investigated separately.

How can we use these technologies to assess the clinical impact of heterogeneity? Despite the wealth of experimental data and computational analyses performed, we still lack a clear understanding of the parameters that will ultimately need to be measured and integrated to assess the impact of tumor heterogeneity on clinical outcomes, but it seems that current knowledge can be applied to envision a more integrated experimental pipeline to systematically test different hypotheses. At the outset, it seems clear that a critical point will be to improve sample handling from collection, through processing and into proper allocation towards different assays.

Other questions need to be carefully considered in design of experiments that aim to study the clinical impact of tumor heterogeneity and evolution. First, which tumor type should be chosen? Tumors need to be relatively large (to provide enough material for the

various assays) and should be readily resectable and progress quickly enough to make a reasonable timeline possible. Second, when and how many times should a tumor be sampled? Third, which parts of the tumor should be analyzed, and should analysis information about its metastatic sites?

Ideally, a coordinated effort to produce this type of sample would generate gold-standard data sets from a large number of patients with well-annotated clinical histories and comprehensive tumor imaging. Each patient's tumor could then be analyzed with a wide array of experimental techniques that provide information about degrees of heterogeneity. The core group of methods should include multifocal bulk and single-cell DNA sequencing, single-cell RNA-seq, multifocal bulk and single-cell mapping of epigenetic marks and single-cell CyTOF-based analysis of candidate marker proteins. Both tumor and microenvironment, including tumor-infiltrating leukocytes, would ideally be assayed. These data could be complemented with data from other assays to measure spatial heterogeneity or investigate cell-free DNA or circulating tumor cells. These assays would provide detailed protein, genome and RNA maps, but to reconstruct patient-specific regulatory networks, algorithms will need to be substantially improved.

This wealth of data should then be made available to the research community to develop methods that analyze and integrate the information provided by different assays to predict disease outcome and therapeutic success, so that researchers can continue to gain insight into the importance and impact of cellular heterogeneity.

Closing remarks

One take-home message from this meeting was that the phenomenon of tumor heterogeneity is likely to influence—for some time to come—all aspects of cancer research, including how tumor biology is perceived, how techniques to study tumors are developed and how patients are treated. This conference was unique in its goal of identifying questions rather than answers, and we hope that this description of 'known unknowns' identified by this small group of experts sparks research and collaboration in the community at large.

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The authors declare competing financial interests: details are available in the online version of the paper.

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