Next-Generation Pathology—Surveillance of Tumor Microecology

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Abstract

A tumor is a heterogeneous population of cells that provides an environment in which every cell resides in a microenvironmental niche. Microscopic evaluation of tissue sections, based on histology and immunohistochemistry, has been a cornerstone in pathology for decades. However, the dawn of novel technologies to investigate genetic aberrations is currently adopted in routine molecular pathology. We herein describe our view on how recent developments in molecular technologies, focusing on proximity ligation assay and padlock probes, can be applied to merge the two branches of pathology, allowing molecular profiling under histologic observation. We also discuss how the use of image analysis will be pivotal to obtain information at a cellular level and to interpret holistic images of tissue sections. By understanding the cellular communications in the microecology of tumors, we will be at a better position to predict disease progression and response to therapy.

Introduction

Progress is not a process that proceeds with a steady pace; it takes leaps when opportunities arise. In science, these leaps spur out of novel views that contradict existing paradigms or emerging technologies, which enable new types of analysis. In biological and medical research, new instruments and technologies have been key elements to explore unknown territories. The development of the microscope provided researchers with the ability to view microorganisms and cells, which has been instrumental for the understanding of how we humans and all other organisms are built up. The ability to visualize the micro-cosmos incited the development of dyes that stain cells and make them easier to see and targeted reagents to selectively visualize cells that share certain features, such as expression of a specific protein. Immunohistochemistry and immunofluorescence that are utilizing enzyme or fluorophore-labeled antibodies targeted against specific proteins were developed to selectively stain cells or tissue sections in order to evaluate expression levels of the targeted protein in individual cells. With the addition of more and more antibodies, we now have a map of the human tissue proteome [1]. Microscopic analysis is a cornerstone in the field of pathology and is routinely used by pathologists to provide diagnosis. Even today, immunohistochemistry is the ideal method to classify precise lineage affiliations and, sometimes, also to define targeted therapeutic options. Another example on how a technology has spurred our increasing knowledge in biology and medicine is DNA-based technologies, such as PCR and sequencing [2,3]. Having a detailed map of the human proteome, as well

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as the tool to interrogate the genome of individual patients, has allowed identification of genomic aberrations in inherited genetic disorders and somatic diseases, such as cancer. These types of analysis are currently being adopted in routine diagnostics with extended impact on relevant therapeutic decisions. The two examples mentioned above differ in spatial resolution: while analysis by microscopy provides information resolved to single cells, sequencing of DNA obtained from a pool of cells or a piece of tissue provides an average measurement over all cells in the sample. They differ also in other aspects: immunohistochemistry is still evaluated by skilled pathologists and mainly performed in singleplex, that is, staining with one antibody per slide, while sequencing is highly automated and performed at extreme multiplexing, up to whole genome sequencing.

We are now approaching a point in history when we will have the opportunity to take another great leap forward. Here we will discuss how microscopic analysis can be performed at greater levels of multiplexing, incorporating genotyping and evaluation of expression levels of both proteins and mRNAs and also providing possibilities to interrogate protein interactions and post-translational modifications (PTMs), hence also monitoring signal pathway activity status. Furthermore, utilizing automated image analysis will provide pathologists with tools to comprehend the vast complexity of data and also enable comparisons of how images are interpreted at different laboratories and will validate the diagnosis of each individual patient.

The Tumor Microenvironment

The interplay between the different cell types in a multicellular organism has been shaped during millions of years of evolution. Each organ and tissue in a human presents a unique microenvironment in which the different cell types reside in highly specialized niches. The microecology in a tumor is not a static situation and how well a cell adapts to changes in environment will have consequences on differentiation, growth and survival of the cells.

Analogies toward the ecology in nature can be a way to approach the situation *in vivo*, although we are aware that this may oversimplify the complexity of the problem. It is, however, an approach that helps understanding tumorigenesis and how cancer cells respond to, and shape, their microenvironment. Cancer can be seen as evolution of a new species that tries to occupy a new microenvironmental niche and will be subjected to Darwinian selection during this process [4]. Theoretically a more immature cell type, with less epigenetic regulation, is more capable of differentiating into a phenotype that can survive and prosper in a new microenvironment [5,6]. As an analogy, the introduction of rabbits in Australia provided them with a compatible environment but within a new ecological context: without predators to control the population. This allowed them to proliferate rapidly. Such opportunistic and ecology-dependent behavior can also be seen for cells under some "spectacular" experimental conditions where transplantation of embryonic stem cells out of context results in teratoma formation [7]. Introduction of teratoma cells into blastocysts can produce healthy mosaic mice [8]. Teratomas are tumors originating in germ cells and have the capacity to differentiate in tissues from three germ cell lineages—endoderm, mesoderm and ectoderm. The ecologic dependence is of particular interest for stem cell therapy where similar problems do occur, for instance, occurrence of teratomas [9,10]. There are also several reports on the maintenance of malignant cells in individuals that do not have clinical symptoms, indicating that they may be kept in control by the microecology [11]. The microenvironmental control of cellular fate, including immune cells in the microenvironment, is a field that very likely will have huge impacts on future oncology, including new cancer treatment modalities [12,13].

As cancer cells proliferate, they compete with normal cells for both space and access to nutrients, shaping the microenvironment to their needs. Cancer cells promote angiogenesis through secretion of pro-angiogenic growth factors that stimulate sprouting of endothelial cells from nearby vessels or by co-opting pre-existing vasculature [14]. Vessels are required to support the cancer cells with oxygen and nutrients, represent a route for metastasis and provide a niche for cancer stem cells [15]. However, tumor vasculature is often malfunctioning and hyper-permeable, leading to poor oxygenation and high interstitial fluid pressure in the tumor [16]. Low oxygen tension in combination with extracellular matrix components and cytokine production in the over-populated microenvironment may induce cancer cells to undergo epithelial-to-mesenchymal transition: a process in which they loose their cell–cell adhesion and polarity to acquire migratory traits for the invasion of surrounding tissues [17,18]. They can then migrate through the surrounding tissue or utilize blood vessels or the lymphatic system as rivers to rapidly reach other sites. To achieve vascular invasion (intravasation), cancer cells tend to migrate in clusters, a process known as collective migration [19]. Most cancer cells succumb in circulation, mostly if they travel as isolated cells, but some are washed up on nearby or distal shores. In fact, traveling and homing to distant sites will depend on clustering of cells in circulation to be successful [20]. Again, microenvironment seems to play a crucial role all along the process of carcinogenesis and cancer progression, even at the very level of cell–cell interactions. In agreement with this, it has been reported that peritoneovenous shunts, draining intractable carcinomatous ascites directly

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from the peritoneum to the venous system, were not necessarily very successful to generate metastasis despite the large amount of malignant cells introduced in circulation [21]. When the cells exit from circulation, extravasation followed by mesenchymal-to-epithelial transition is needed to revert to their original phenotype. As the cancer cells have evolved in a specific microecology, in the site of the primary tumor, their chance of surviving a journey to an adjacent site of the same organ is better than if they encounter a more different microenvironment. It has been suggested that cancer can grow in the primary site through homing to the same organ in a process called self-seeding [22]. It has also been suggested that cancer cells try to reshape the microenvironment of distant organs by secreting exosomes containing proteins and RNA that affect the phenotype of the retrieving cells [23]. Once the cancer cells have succeeded in this, they may proliferate and form metastasis.

Cancer Development

The development of a cancer is in most cases a stepwise process, requiring mutations in several oncogenes and tumor suppressor genes [24], together with epigenetic alterations. Although these changes alter the phenotype of the cancer cells, they still carry most of the epigenetic signature from the differentiation lineage they derived from, and hence, the properties of different types of cancer differ depending on origin and mutational spectrum [25]. Exceptions are cancers derived from metaplasia, a transdifferentiation of an adult tissue into a different adult tissue but in a mislocalized setting. In these cases, a differentiation reprogramming occurs before carcinogenesis or at very early cancer stages [26,27]. Over the last decades, this information has been used to further subgroup different classes of cancers that share phenotypic and genetic features. The more parameters used for the grouping, the more subgroups will emerge. Also, the heterogeneity of the details makes it difficult to evaluate the prognostic and therapeutic implications since large multicentric studies will be necessary to attain statistical significance. At the limit, when taking into account the unique genetic makeup of each individual, the potential influence of the tumor microenvironment and the mutational and epigenetic status of the cancer cells, all tumors will be unique. No patient will be identical with another, although they will share some features. In addition, the tumors often consist of several subclones with shared and unique mutations [28], which imply that even a single tumor will contain cancer cells with different cellular programs and abilities to interact with the microenvironment, utilizing different niches or competing with other clones within a niche [4].

Most treatment options for cancer cells aim at killing proliferating cells, for example, by disturbing replication or targeting a signaling pathway to which the cancer cells have become addicted to, due to activating mutations omitting the need for upstream signals. Although initial response may be very prominent, the cancer cells will often develop resistance and come back with a vengeance [29]. With the more recent use of targeted therapy, we have seen that cells can acquire novel mutations in order to evade succumbing to therapy. Alternatively, the alterations in the microecology caused by the treatment, that is, elimination of sensitive clones, create an opportunity for subclones harboring advantageous mutations to expand and fill the now empty niche or that heterogeneity in transcription facilitates survival of subpopulations [30]. However, another explanation is that the cancer cells rewire the intracellular networks to circumvent the blockage at the targeted node [31,32]. The escape mechanisms are not well understood and future pathology will need to monitor how treatment affects both the cancer cells and the microecology in the tumors, namely, the variable composition in terms of different sorts of pro- and anti-tumoral lymphocytes and macrophages. The inflammatory response brought by a severe killing of cancer cells may also stimulate quiescent subclones with a more stem-cell-like phenotype, so called cancer-initiating cells, and their progeny will be able to fill the void in the tumor microecology created by the treatment. It is possible that the strategy of eradicating the cancer cells fuels tumor evolution, and alternative approaches to control the microecology and block the proliferation of cancer cells, without killing them, would be a substitute. High content analysis will be essential in order to understand the tumor ecology, providing information on what signaling pathways are active in different cell types in a tissue and how this correlate with cell–cell communication and genotype. It is clear that complexity will increase, by the gain in insight both at the cancer cell and at the microenvironment levels, and that a coordinated view on the whole network of intercellular communication will be needed.

Method Requirements for Analysis of Tumors

There are four desirable key features of methods for surveillance of tumor microecology. First of all is multiplexing: in parallel, many different types of information are needed to decode the delicate interplay between cells. Second, analytical methods have to target functional aspects of the signaling networks—such as protein–protein interactions, PTMs of proteins and expression of target genes. Third, the methods will have to be able to determine the genotypes, identifying a spectrum of different mutations. Finally, the methods should be spatially

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resolved and retain information on tumor architecture in order to determine intratumor heterogeneity and intercellular and intracellular signaling.

From the early days of immunohistochemistry, pathologists know that many tumor markers are expressed in the invasive front of tumors and modulate matrix remodeling. These modifications have recently been ascribed to the process of epithelial-to-mesenchymal transition. For nucleic acids, it is currently possible to perform single cell analysis of multiple targets, including sequencing of whole transcriptomes from individual cells [33]. For proteomics, however, this is a much more challenging task, as efficiency of both mass spectrometry and affinity-based technologies is far from detecting single molecules. The throughput is a limiting factor using microdissected samples; currently, analysis of thousands of cells would not be practical. To achieve this, it may be more straightforward to perform the analysis directly on the tissue sections, using non-targeted approaches, that is, imaging mass spectrometry [34,35], or targeted approaches, utilizing labeled antibodies for example. The number of different fluorophores that simultaneously can be visualized is limited due to overlaps in excitation and emission spectra of the different fluorophores used. A way to increase the number of measured parameters is to use antibodies labeled with mass tags [36], expanding the number of possible measure points in a stained tissue [37,38]. Another way to increase multiplexing is to perform multiple staining with sequential readout. For this approach, the analysis has to be non-destructive; hence, mass cytometry could not be used. Instead, one can rely on analysis using fluorescence microscopy for example. By performing repetitive cycles of staining, imaging and bleaching, with the use of a single fluorophore to label all the different antibodies, we can perform highly multiplexed analysis [39]. With the use of this strategy, the number of analyzed parameters will increase linearly, reflecting the number of cycles performed and the number of fluorophores used. To increase multiplexing even further, with a concomitant reduction of number of performed cycles, a strategy is to barcode the different molecular objects identified, using the information obtained in each cycle providing an exponential increase of identities, that is, number of fluorophores to the power of the number of cycles. The four different bases investigated at each position will be reported with a unique fluorophore by utilizing sequencing *in situ* to decode identities. Hence, sequencing of four bases will obtain $4^4 = 256$ different signatures [40]. The different strategies to increase multiplexing are heavily dependent on advanced image analysis approaches and will be described further in subsequent section.

The next methodological hurdle to detect signaling network activity is to target protein interactions and PTMs. The cellular signaling network relays information via protein–protein interactions. All proteins have the ability to bind all others, which interactions that are most present depend on affinities and local concentration [41]. As a consequence of functional interactions, changes in conformation may occur due to PTMs or complex formation with partner proteins. This will change their affinity toward other proteins, determining the possibility to engage in further/novel protein interactions. Translocation of the proteins in active conformation to other compartments of the cell, for example, from the plasma membrane to the nucleus, will also have consequences on which interactions may occur, as the local concentration of interaction partners differ in between cellular compartments. Which protein–protein interactions are present in a cell, the amounts and location can be seen as the interface between the genetic/epigenetic composition of the cell and external cues, reflecting the activity status of the cells. There is a large repertoire of affinity reagents targeting PTMs, and these reagents can be used to investigate whether, or not, a protein is in a functionally active conformation. By using a panel of such reagents, it is possible to determine functional states of individual cells within a population. However, single recognition assays suffer from problems with cross-reactivity. All affinity reagents are destined to bind multiple targets, with different affinities. To increase selectivity, we can use assays based on dual, or multiple, recognition events. An additional advantage with such a strategy is that it also facilitates analysis of protein complexes, where two or more affinity reagents bind to different proteins within a protein complex. There are several ways to determine dual binding events. The most straightforward way is to label the different affinity reagents with unique fluorophores and determine colocalization of the staining. However, this will require very sophisticated microscopes operating below diffraction limit with sensitivities to detect single fluorophores. At present, this is extremely challenging to perform on tissue sections. An alternative approach is to use the proximal binding as a functional requirement to generate a signal. This strategy is used in the Förster resonance energy transfer in which a donor fluorophore only can transfer the energy to an acceptor fluorophore if they are in close proximity, within a few nanometers. By determining emission spectra from donor and acceptor, or change in lifetime, we can record proximity between fluorophore-labeled antibodies [42].

Methods for Surveillance of Tumor Microecology

A method that retains the dependency of proximal binding of antibodies and provides a mean for signal amplification is the *in situ* proximity ligation assay (PLA) [43] (Fig. 1a) (for review of different designs, see Koos *et al.* [44]). This technology utilizes DNA

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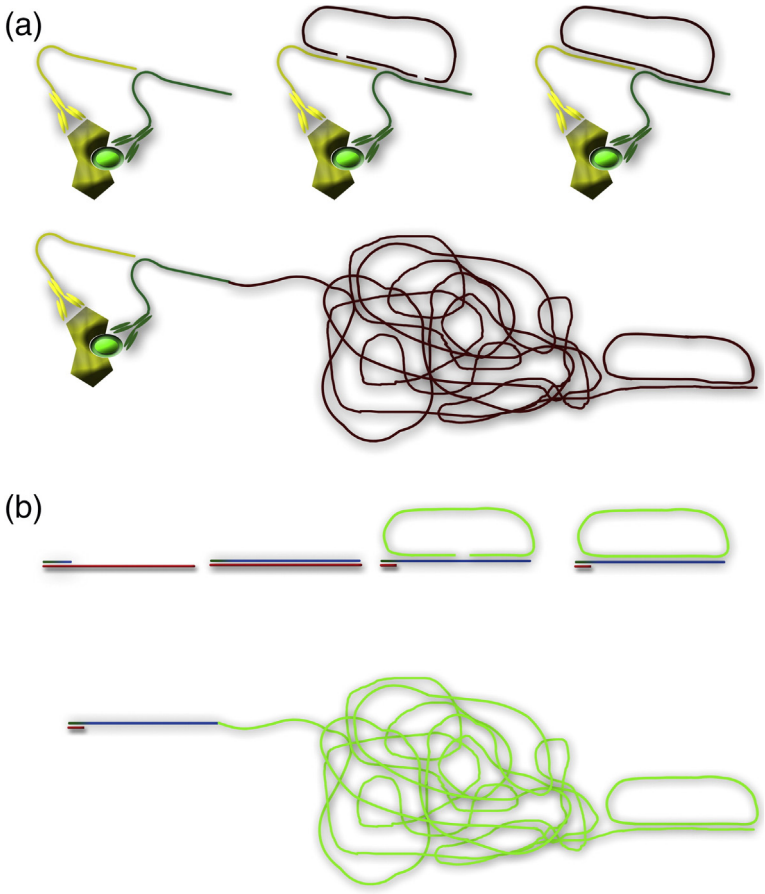

Fig. 1. Principle of *in situ* PLA and padlock probes. (a) For detection of protein–protein interactions, the *in situ* PLA requires two proximity probes to bind to their respective targets. If the probes bind in close proximity, two circularization oligonucleotides can be hybridized and ligated in order to form a circular DNA molecule. In the ensuing RCA, this DNA molecule can be amplified as an extension of one of the proximity probes. The so-generated RCA product can be visualized by hybridization of fluorophore-labeled detection oligonucleotides. (b) In order to detect RNA molecules via padlock probes, a primer oligonucleotide that partly consists of locked nucleic acids needs to hybridize to the RNA molecule. This primer will be extended in an *in situ* reverse transcription upon which the RNA part of the DNA/RNA hybrid is degraded and a padlock probe is hybridized to the cDNA. Importantly, the RNA bound to the locked nucleic acids part of the primer is not degraded, providing a link to the rest of the RNA molecule. The padlock probe is then ligated onto the target and amplified using RCA. Visualization of the RCA product is achieved by hybridization of fluorophore-labeled detection oligonucleotides.

oligonucleotides conjugated to antibodies (proximity probes) to provide a template for ligation of subsequently added circularization DNA oligonucleotides. The ligation will only generate circular products if the proximity probes bind in close proximity. The length and orientation of the oligonucleotides, as well as the size of the affinity reagents, determine the distance requirement for *in situ* PLA. Once a circular ligation product is formed, it can be amplified by rolling circle amplification (RCA). The DNA oligonucleotide on one of the proximity probes will act as a primer for RCA and the RCA product will hence be an elongation of that proximity probe, a concatemer of several hundreds of repeats, complementary to the circular ligation product. By hybridizing fluorophore-labeled detection DNA oligonucleotides to the RCA product, we will stain each single molecule with several hundred fluorophores. The signal amplification provided by RCA facilitates detection of single ligation events. However, the size of an RCA product is approximately 1 μm in diameter, which limits the number of events that can be recorded in a single cell. To increase the dynamic range, we can use alternative circularization oligonucleotides in a set ratio and can detect them by different fluorophore-labeled detection oligonucleotides [45]. When one identity saturates the cell, a more diluted one will be analyzable. *In situ* PLA can be used for visualization of endogenous protein–protein interactions [43], PTMs [46–49] and protein–nucleic acid interactions [50–52], and the generation of discrete signals facilitates enumeration by digital image processing and analysis. The ability to determine protein interactions and PTMs in fixed tissue sections may facilitate the use in routine pathology. A specific application where *in situ* PLA has successfully overcome a technical limitation is the identification of mucin glycoforms [53], the major cancer biomarkers used in the clinical setting. In fact, till recently, the protein components and the glycan components of mucins were impossible to get into a compound glycoprotein profile. Moreover, the combined profile is giving hope for increasing sensitivity and specificity to the classical approaches. Other examples are EGFR/HER interactions [54–56] and PDGFR phosphorylation [57,58]. It has to be mentioned that *in situ* PLA is a targeted approach, in pairwise interactions that are monitored. Selecting potential interaction partners relies on work performed on protein–protein interaction networks by other methods that are more suitable for screening purposes, such as two-hybrid assays [59–61].

For analysis of nucleic acids, the stringent requirement of circularizing can be used to provide

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single nucleotide discrimination. Padlock probes are linear DNA oligonucleotides designed so that hybridization of a padlock probe with its target sequence will bring the 5′- and the 3′-ends of the padlock probe into juxtaposition [62] (Fig. 1b). Only correctly bound padlock probes would thus be possible to be ligated into a circular conformation, such that a mismatch in the junction will abolish, or prominently reduce, the ligation efficiency. Double-stranded DNA has to be converted into single-stranded DNA using, for instance, restriction enzymes and exonucleases prior to padlock probing, in order to allow hybridization of a padlock probe to a DNA target [63]. To probe RNA molecules, we require the use of reverse transcriptase to generate cDNA templates for hybridization [64]. Once the padlock probe has been ligated into a circular conformation, its ligation template (i.e., the single-stranded DNA to which the padlock probe hybridized to) can act as a primer for RCA to generate a localized product that can be visualized with hybridization of fluorophore-labeled detection oligonucleotides. The method can be used to genotype mRNA [65]. As both *in situ* PLA and padlock probes utilize RCA to generate an amplified signal, the two methods can be combined to provide measurements of all steps in signaling networks [66,67], that is, from surface receptors down to expression of target genes. In addition, both methods are well suited for multiplexing [40,68] and can be read out by serial hybridization of multiple fluorophore-labeled detection oligonucleotides or via *in situ* sequencing. *In situ* sequencing of padlock probes has been used for highly multiplexed analysis of mRNA expression [40,69].

Retrieving Information from Images

Visually interpreting the histology of a tissue section requires years of training, and the value of experience is of uttermost importance. However, the evaluation is difficult and sometimes biased, and pathologists often disagree on diagnosis [70]. A major advantage of classical pathologic examination is that it takes into account gross histologic characteristics of a tumor that sum up a huge amount of minor molecular and topographically restricted characteristics. If a tumor is macroscopically or microscopically invading the serosa of a gastrointestinal specimen, this gives relevant, bulk information, whatever the mutational profile of the specific tumor might be. Even microscopic studies at the cellular level are, at this moment, comparably gross and maybe also highly informative because of this: if a tumor is invading veins, this signals hematogeneous dissemination with a high probability, again whatever the molecular profile of the case under scrutiny is. Pathology has, however, several limitations since it is difficult to monitor a large number of cells, and individual cells may easily be missed. Hence, automated image analysis will provide an important complement for the pathologists in the evaluation of tissue sections. It will provide a tool for better characterization of the tissue and for the identification of individual cells of defined phenotypes to complement and not compete with the extremely useful observations at a more “macro” level. Morphological features associated with cell state include, for example, cell size, nucleus-to-cytoplasm ratio and chromatin characteristics including granularity, margination, condensation and compaction. These function/morphology relationships have been extensively used in analyses of malignant cells [71]. It has also been shown that these features can be captured with high stringency using automated digital image analyses [72]. In addition, digital image processing and analysis provides tools for initial characterization of tissue samples detecting low-resolution characteristics such as vascularization [73], gland shape and distribution, tumor shape and smoothness of the tumor–host interface [74].

Future Perspectives

With morphologic features and molecular profiles as input, we can group tissue areas and cells with common characteristics together using spatially resolved high-dimensional clustering [75,76]. Combining more and more features in the analysis, more and more cell types and functional states can be identified, again without loosing the organizational level given by classical pathologic examination. Instead of discrete classes of cell populations, the analysis will produce a continuum of clusters linked by the differentiation lineage from where they arose. This type of analysis has the potential to determine the functional state of each individual cell and will be essential to detect, for example, single cancer cells invading the surrounding stroma. However, it will not be able to discern what signals underlay these functional states, that is, to what extent is the functional states regulated by the genomic/epigenomic blueprint *versus* cell–cell contacts and secreted factors from adjacent cells. When the spatial information is taken into account, we can also discern the functional states of the surrounding cells. Knowing the functional state of each cell together with the architecture of the tissue is a prerequisite to perform analysis of cellular communication, which is the basis for understanding microecology. Further, by combining analysis of signaling pathway activity status with genotyping, that is, using padlock probes, it will be feasible also to determine what features different subclones exhibit and how/if this is altered when the microecology is affected by chemotherapy. This will generate topographically oriented information on top of molecular and mutational data, giving coherence and compactness to our current vision on microecology of tumor tissue and its neighborhood relationships, in the absence or in the presence of therapeutic interventions.

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As previously mentioned, an advantage of *in situ* PLA and padlock probes is that RCA generates bright discrete objects that can be enumerated using image analysis [77]. For *in situ* sequencing, the spatial localization of each individual RCA product, possibly by using cellular staining as reference points, has to be determined in order to decode the fluorescence identity of every RCA products in a set of serial image acquisitions [78]. After a set of images has been acquired on tissue sections stained with *in situ* PLA and padlock probes, the tissue sections can then be stained with conventional immunohistofluorescence/immunohistochemistry and hematoxylin/eosin staining and new sets of images can be recorded. All these different types of images, obtained by fluorescence and brightfield microscopy using several fields of view stitched up to larger images, have to be lined up correctly in order to produce an image that contains all the information in multiple image channels (Fig. 2). Apart from detection of RCA products, the image also has to be partitioned into cells and other structures of interest, such as blood vessels and glands. Information linked to these structures, such as shape, intensity and texture, needs to be retrieved and processed, for example, using machine learning and spatial clustering methods, to determine cell types and functional states.

Finally, all the gathered information, including cell identities, can be visualized using pseudo-colors or by representing different types of RCA products by color- and shape-coded markers. The different types of information can then be visualized using a viewer in analogy with software to visualize satellite images combined with street maps and traffic monitoring, for example, Google Earth. The next step in the image-processing pipeline is to determine the consequence a physical interaction, or close proximity between cells, has on the molecular profiles, or functional states, of the cells. Mapping interactions between cells with defined molecular profiles will be a similar task as mapping protein interactions in systems biology. This will provide information on cellular communications; that is, it will determine how the molecular profiles of cells are dependent on the cell type lineage and on the molecular profile of its neighbors. Utilizing information on genotypes and functional states of signaling networks, while keeping the spatial information of each cell, will give pathologists and researchers the possibility to explore tumor microecology. One fantastic possibility with this approach is that it generates images with several layers of information, giving context and momentum to minute molecular events and comprehensive deepness to "gross" pathological characteristics.

Open-access libraries of analyzed tissue sections combining molecular profiling and with morphological analysis by image analysis will provide valuable reference materials for pathologists. The methods and pipelines described herein will not replace pathologists, but rather, these will help them in their work and improve the analysis and decision-making procedure. An image/knowledge library together with efficient visualization and data exploration tools would allow identification of features shared among different patients, together with information on the outcome, which will be important to tailor the treatment strategies for new patients.

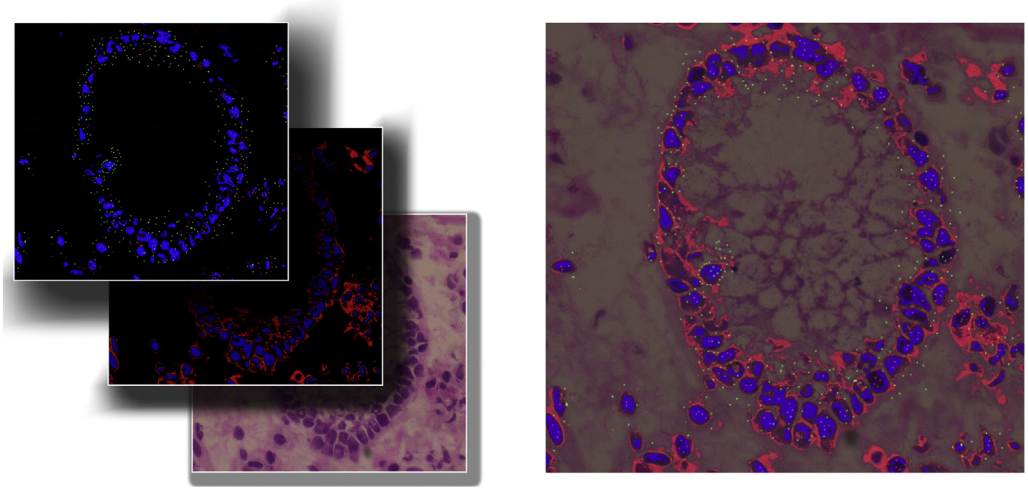

Fig. 2. By overlaying pictures taking from hematoxylin/eosin staining and immunofluorescence with these obtained with *in situ* PLA and padlock probes, we will be able to achieve a high level of information about different cell types within a tissue. Hematoxylin/eosin staining will aid in evaluating morphology, and immunofluorescence might be used to distinguish different cell types by expression of marker proteins. *In situ* PLA will give insights into protein interactions inside the cells while padlock probes will provide valuable information about expression of RNA transcripts and presence of mutations. All this information can be combined in order to segment cells and identify populations of cells within shared features.

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PTM, post-translational modification; PLA, proximity ligation assay; RCA, rolling circle amplification.

References

[1] Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Proteomics. Tissue-based map of the human proteome. Science 2015;347:1260419.
[2] Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harb Symp Quant Biol 1986;51:263–73.
[3] Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 1977;74:5463–7.
[4] Greaves M, Maley CC. Clonal evolution in cancer. Nature 2012;481:306–13.
[5] Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. Nat Rev Genet 2006;7:21–33.
[6] Wu MZ, Tsai YP, Yang MH, Huang CH, Chang SY, Chang CC, et al. Interplay between HDAC3 and WDR5 is essential for hypoxia-induced epithelial–mesenchymal transition. Mol Cell 2011;43:811–22.
[7] Lensch MW, Schlaeger TM, Zon LI, Daley GQ. Teratoma formation assays with human embryonic stem cells: a rationale for one type of human–animal chimera. Cell Stem Cell 2007;1:253–8.
[8] Illmensee K, Mintz B. Totipotency and normal differentiation of single teratocarcinoma cells cloned by injection into blastocysts. Proc Natl Acad Sci U S A 1976;73:549–53.
[9] Cunningham JJ, Ulbright TM, Pera MF, Looijenga LH. Lessons from human teratomas to guide development of safe stem cell therapies. Nat Biotechnol 2012;30:849–57.
[10] Fong CY, Gauthaman K, Bongso A. Teratomas from pluripotent stem cells: a clinical hurdle. J Cell Biochem 2010;111:769–81.
[11] Rich AR. On the frequency of occurrence of occult carcinoma of the prostrate. 1934. Int J Epidemiol 2007;36:274–7.
[12] Bissell MJ, Hines WC. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. Nat Med 2011;17:320–9.
[13] Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med 2013;19:1423–37.
[14] Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature 2011;473:298–307.
[15] Catalano V, Turdo A, Di Franco S, Dieli F, Todaro M, Stassi G. Tumor and its microenvironment: a synergistic interplay. Semin Cancer Biol 2013;23:522–32.
[16] Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. Nat Rev Drug Discov 2011;10:417–27.
[17] Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial–mesenchymal transitions in development and disease. Cell 2009;139:871–90.
[18] Jung HY, Fattet L, Yang J. Molecular pathways: linking tumor microenvironment to epithelial–mesenchymal transition in metastasis. Clin Cancer Res 2015;21(5):962–8.
[19] Friedl P, Hegerfeldt Y, Tusch M. Collective cell migration in morphogenesis and cancer. Int J Dev Biol 2004;48:441–9.
[20] Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, et al. Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. Cell 2014;158:1110–22.
[21] Tarin D, Price JE, Kettlewell MG, Souter RG, Vass AC, Crossley B. Clinicopathological observations on metastasis in man studied in patients treated with peritoneovenous shunts. Br Med J (Clin Res Ed) 1984;288:749–51.
[22] Comen E, Norton L, Massague J. Clinical implications of cancer self-seeding. Nat Rev Clin Oncol 2011;8:369–77.
[23] Peinado H, Aleckovic M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med 2012;18:883–91.
[24] Sjoblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, et al. The consensus coding sequences of human breast and colorectal cancers. Science 2006;314:268–74.
[25] Eirew P, Steif A, Khattra J, Ha G, Yap D, Farahani H, et al. Dynamics of genomic clones in breast cancer patient xenografts at single-cell resolution. Nature 2014;518:422–6.
[26] Camilo V, Garrido M, Valente P, Ricardo S, Amaral AL, Barros R, et al. Differentiation reprogramming in gastric intestinal metaplasia and dysplasia: role of SOX2 and CDX2. Histopathology 2015;66:343–50.
[27] Slack JM. Metaplasia and somatic cell reprogramming. J Pathol 2009;217:161–8.
[28] Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 2012;366:883–92.
[29] Gillies RJ, Verduzco D, Gatenby RA. Evolutionary dynamics of carcinogenesis and why targeted therapy does not work. Nat Rev Cancer 2012;12:487–93.
[30] Lee MC, Lopez-Diaz FJ, Khan SY, Tariq MA, Dayn Y, Vaske CJ, et al. Single-cell analyses of transcriptional heterogeneity

during drug tolerance transition in cancer cells by RNA sequencing. Proc Natl Acad Sci U S A 2014;111:E4726–35.
[31] Romano D, Nguyen LK, Matallanas D, Halasz M, Doherty C, Kholodenko BN, et al. Protein interaction switches coordinate Raf-1 and MST2/Hippo signalling. Nat Cell Biol 2014;16:673–84.
[32] Tran KA, Jackson SA, Olufs ZP, Zaidan NZ, Leng N, Kendziorski C, et al. Collaborative rewiring of the pluripotency network by chromatin and signalling modulating pathways. Nat Commun 2015;6:6188.
[33] Wu AR, Neff NF, Kalisky T, Dalerba P, Treutlein B, Rothenberg ME, et al. Quantitative assessment of single-cell RNA-sequencing methods. Nat Methods 2014;11:41–6.
[34] Caprioli RM, Farmer TB, Gile J. Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. Anal Chem 1997;69:4751–60.
[35] Stoeckli M, Chaurand P, Hallahan DE, Caprioli RM. Imaging mass spectrometry: a new technology for the analysis of protein expression in mammalian tissues. Nat Med 2001;7:493–6.
[36] Bendall SC, Simonds EF, Qiu P, Amir el AD, Krutzik PO, Finck R, et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. Science 2011;332:687–96.
[37] Angelo M, Bendall SC, Finck R, Hale MB, Hitzman C, Borowsky AD, et al. Multiplexed ion beam imaging of human breast tumors. Nat Med 2014;20:436–42.
[38] Giesen C, Wang HA, Schapiro D, Zivanovic N, Jacobs A, Hattendorf B, et al. Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. Nat Methods 2014;11:417–22.
[39] Schubert W, Bonnekoh B, Pommer AJ, Philipsen L, Bockelmann R, Malykh Y, et al. Analyzing proteome topology and function by automated multidimensional fluorescence microscopy. Nat Biotechnol 2006;24:1270–8.
[40] Ke R, Mignardi M, Pacureanu A, Svedlund J, Botling J, Wahlby C, et al. *In situ* sequencing for RNA analysis in preserved tissue and cells. Nat Methods 2013;10:857–60.
[41] Schmick M, Bastiaens PI. The interdependence of membrane shape and cellular signal processing. Cell 2014;156:1132–8.
[42] Bastiaens PI, Jovin TM. Microspectroscopic imaging tracks the intracellular processing of a signal transduction protein: fluorescent-labeled protein kinase C beta I. Proc Natl Acad Sci U S A 1996;93:8407–12.
[43] Soderberg O, Gullberg M, Jarvius M, Ridderstrale K, Leuchowius KJ, Jarvius J, et al. Direct observation of individual endogenous protein complexes *in situ* by proximity ligation. Nat Methods 2006;3:995–1000.
[44] Koos B, Andersson L, Clausson CM, Grannas K, Klaesson A, Cane G, et al. Analysis of protein interactions *in situ* by proximity ligation assays. Curr Top Microbiol Immunol 2014;377:111–26.
[45] Clausson CM, Allalou A, Weibrecht I, Mahmoudi S, Farnebo M, Landegren U, et al. Increasing the dynamic range of *in situ* PLA. Nat Methods 2011;8:892–3.
[46] Jarvius M, Paulsson J, Weibrecht I, Leuchowius KJ, Andersson AC, Wahlby C, et al. *In situ* detection of phosphorylated platelet-derived growth factor receptor beta using a generalized proximity ligation method. Mol Cell Proteomics 2007;6:1500–9.
[47] Conze T, Carvalho AS, Landegren U, Almeida R, Reis CA, David L, et al. MUC2 mucin is a major carrier of the cancer-associated sialyl-Tn antigen in intestinal metaplasia and gastric carcinomas. Glycobiology 2010;20:199–206.
[48] Leuchowius KJ, Weibrecht I, Landegren U, Gedda L, Soderberg O. Flow cytometric *in situ* proximity ligation analyses of protein interactions and post-translational modification of the epidermal growth factor receptor family. Cytometry A 2009;75:833–9.
[49] Leuchowius KJ, Jarvius M, Wickstrom M, Rickardson L, Landegren U, Larsson R, et al. High content screening for inhibitors of protein interactions and post-translational modifications in primary cells by proximity ligation. Mol Cell Proteomics 2010;9:178–83.
[50] Weibrecht I, Gavrilovic M, Lindbom L, Landegren U, Wahlby C, Soderberg O. Visualising individual sequence-specific protein–DNA interactions *in situ*. N Biotechnol 2012;29:589–98.
[51] Gomez D, Shankman LS, Nguyen AT, Owens GK. Detection of histone modifications at specific gene loci in single cells in histological sections. Nat Methods 2013;10:171–7.
[52] Jung J, Lifland AW, Zurla C, Alonas EJ, Santangelo PJ. Quantifying RNA–protein interactions *in situ* using modified-MTRIPs and proximity ligation. Nucleic Acids Res 2013;41:e12.
[53] Ricardo S, Marcos-Silva L, Pereira D, Pinto R, Almeida R, Soderberg O, et al. Detection of glyco-mucin profiles improves specificity of MUC16 and MUC1 biomarkers in ovarian serous tumours. Mol Oncol 2014;9:503–12.
[54] Aubele M, Spears M, Ludyga N, Braselmann H, Feuchtinger A, Taylor KJ, et al. *In situ* quantification of HER2-protein tyrosine kinase 6 (PTK6) protein–protein complexes in paraffin sections from breast cancer tissues. Br J Cancer 2010;103:663–7.
[55] Spears M, Taylor KJ, Munro AF, Cunningham CA, Mallon EA, Twelves CJ, et al. *In situ* detection of HER2:HER2 and HER2:HER3 protein–protein interactions demonstrates prognostic significance in early breast cancer. Breast Cancer Res Treat 2012;132:463–70.
[56] Smith MA, Hall R, Fisher K, Haake SM, Khalil F, Schabath MB, et al. Annotation of human cancers with EGFR signaling-associated protein complexes using proximity ligation assays. Sci Signal 2015;8:ra4.
[57] Koos B, Paulsson J, Jarvius M, Sanchez BC, Wrede B, Mertsch S, et al. Platelet-derived growth factor receptor expression and activation in choroid plexus tumors. Am J Pathol 2009;175:1631–7.
[58] Paulsson J, Lindh MB, Jarvius M, Puputti M, Nister M, Nupponen NN, et al. Prognostic but not predictive role of platelet-derived growth factor receptors in patients with recurrent glioblastoma. Int J Cancer 2011;128:1981–8.
[59] Petschnigg J, Groisman B, Kotlyar M, Taipale M, Zheng Y, Kurat CF, et al. The mammalian-membrane two-hybrid assay (MaMTH) for probing membrane–protein interactions in human cells. Nat Methods 2014;11:585–92.
[60] Snider J, Kittanakom S, Damjanovic D, Curak J, Wong V, Stagljar I. Detecting interactions with membrane proteins using a membrane two-hybrid assay in yeast. Nat Protoc 2010;5:1281–93.
[61] Fields S, Song O. A novel genetic system to detect protein–protein interactions. Nature 1989;340:245–6.
[62] Nilsson M, Malmgren H, Samiotaki M, Kwiatkowski M, Chowdhary BP, Landegren U. Padlock probes: circularizing oligonucleotides for localized DNA detection. Science 1994;265:2085–8.
[63] Larsson C, Koch J, Nygren A, Janssen G, Raap AK, Landegren U, et al. *In situ* genotyping individual DNA molecules by target-primed rolling-circle amplification of padlock probes. Nat Methods 2004;1:227–32.

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[64] Larsson C, Grundberg I, Soderberg O, Nilsson M. *In situ* detection and genotyping of individual mRNA molecules. Nat Methods 2010;7:395–7.
[65] Grundberg I, Kiflemariam S, Mignardi M, Imgenberg-Kreuz J, Edlund K, Micke P, et al. *In situ* mutation detection and visualization of intratumor heterogeneity for cancer research and diagnostics. Oncotarget 2013;4:2407–18.
[66] Weibrecht I, Grundberg I, Nilsson M, Soderberg O. Simultaneous visualization of both signaling cascade activity and end-point gene expression in single cells. PLoS One 2011;6:e20148.
[67] Weibrecht I, Lundin E, Kiflemariam S, Mignardi M, Grundberg I, Larsson C, et al. *In situ* detection of individual mRNA molecules and protein complexes or post-translational modifications using padlock probes combined with the *in situ* proximity ligation assay. Nat Protoc 2013;8:355–72.
[68] Leuchowius KJ, Clausson CM, Grannas K, Erbilgin Y, Botling J, Zieba A, et al. Parallel visualization of multiple protein complexes in individual cells in tumor tissue. Mol Cell Proteomics 2013;12:1563–71.
[69] Kiflemariam S, Mignardi M, Ali MA, Bergh A, Nilsson M, Sjoblom T. *In situ* sequencing identifies TMPRSS2-ERG fusion transcripts, somatic point mutations and gene expression levels in prostate cancers. J Pathol 2014;234:253–61.
[70] Stenkvist B, Bengtsson E, Eriksson O, Jarkrans T, Nordin B, Westman-Naeser S. Histopathological systems of breast cancer classification: reproducibility and clinical significance. J Clin Pathol 1983;36:392–8.
[71] Gurcan MN, Boucheron LE, Can A, Madabhushi A, Rajpoot NM, Yener B. Histopathological image analysis: a review. IEEE Rev Biomed Eng 2009;2:147–71.
[72] Madabhushi A, Agner S, Basavanhally A, Doyle S, Lee G. Computer-aided prognosis: predicting patient and disease outcome via quantitative fusion of multi-scale, multi-modal data. Comput Med Imaging Graph 2011;35:506–14.
[73] Dubinski W, Gabril M, Iakovlev VV, Scorilas A, Youssef YM, Faragalla H, et al. Assessment of the prognostic significance of endoglin (CD105) in clear cell renal cell carcinoma using automated image analysis. Hum Pathol 2012;43:1037–43.
[74] Heindl A, Nawaz S, Yuan Y. Mapping spatial heterogeneity in the tumor microenvironment: a new era for digital pathology. Lab Invest 2015. http://dx.doi.org/10.1038/labinvest.2014.155.
[75] Sommer C, Straehle C, Kothe U, Hamprecht FA. Ilastik: interactive learning and segmentation toolkit. 2011 8th IEEE International Symposium on Biomedical Imaging: From Nano to Macro; 2011. p. 230–3.
[76] Linderman MD, Bjornson Z, Simonds EF, Qiu P, Bruggner RV, Sheode K, et al. CytoSPADE: high-performance analysis and visualization of high-dimensional cytometry data. Bioinformatics 2012;28:2400–1.
[77] Smal I, Loog M, Niessen W, Meijering E. Quantitative comparison of spot detection methods in fluorescence microscopy. IEEE Trans Med Imaging 2010;29:282–301.
[78] Pacureanu AKR, Mignardi M, Nilsson M, Wählby C. Image based *in situ* sequencing for RNA analysis in tissue. Proc IEEE Int Symp Biomed Imaging 2014:211–5.

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