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Pre-existing Epithelial Diversity in Normal Human Livers: A Tissue-tethered Cytometric Analysis in Portal/Periportal Epithelial Cells

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Abstract

Routine light microscopy identifies two distinct epithelial cell populations in normal human livers: hepatocytes and biliary epithelial cells (BEC). Considerable epithelial diversity, however, arises during disease states when a variety of hepatocyte-BEC hybrid cells appear. This has been attributed to activation and differentiation of putative hepatic progenitor cells (HPC) residing in the Canals of Hering and/or metaplasia of pre-existing mature epithelial cells. A novel analytic approach consisting of multiplex labeling, high resolution whole slide imaging (WSI), and automated image analysis was used to determine if more complex epithelial cell phenotypes pre-existed in normal adult human livers, which might provide an alternative explanation for disease-induced epithelial diversity. “Virtually digested” WSI enabled quantitative cytometric analyses of individual cells displayed in a variety of formats (e.g. scatter plots) while still tethered to the WSI and tissue structure. We employed biomarkers specifically-associated with mature epithelial forms (HNF4α for hepatocytes, CK19 and HNF1β for BEC) and explored for the presence of cells with hybrid biomarker phenotypes. Results showed abundant hybrid cells in portal bile duct BEC, canals of Hering, and immediate periportal hepatocytes. These bi-potential cells likely serve as a reservoir for the epithelial diversity of ductular reactions, appearance of hepatocytes in bile ducts, and the rapid and fluid transition of BEC to hepatocytes, and vice versa.

Conclusion—Novel imaging and computational tools enable increased information extraction from tissue samples and quantify the considerable pre-existent hybrid epithelial diversity in normal human liver. This computationally-enabled tissue analysis approach offers much broader potential beyond the results presented here.

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Disclosure

A.J.D. is a member of the clinical development team of Omnyx, LLC (www.omnyx.com), a corporation developing a complete digital pathology solution. A.L. serves as a consultant for Carl Zeiss MicroImaging, LLC.

Keywords

Digital imaging; progenitor cells; Canals of Hering; hepatocyte nuclear factors; fluorescence microscopy

Introduction

In normal human livers, routine conventional histology divides epithelial cells into hepatocytes and biliary epithelial cells (BEC). A wide variety of hepatocyte-BEC transitional phenotypes, however, quickly appear in diseased livers. Cells with transitional phenotypes are thought to arise from proliferation and differentiation of hepatic progenitor cells (HPC) (1, 2) and/or from metaplasia of mature hepatocytes and BEC(3). Otherwise typical hepatocytes can be found in portal tract bile ducts with no connection to lobular-based hepatocytes(4) and the liver responds “intelligently” to various insults by rapidly producing more BEC and/or hepatocytes, as needed (5, 6). Understanding the complexity of these wound repair responses using traditional light and electron microscopy limits the type of data that can be extracted from tissue samples (5, 6).

Indeed, the “value proposition” of conventional histology is in question because of various shortcomings: tissue biopsies are invasive, expensive, potentially morbidity- and mortality-producing, an unpleasant patient experience, subject to sampling error, and interpretations are prone to bias, subjectivity and inter-observer variability(7) compared to less-invasive and potentially more informative monitoring techniques, such as whole genome sequencing and peripheral blood and fluid mRNA arrays and proteomics/metabolomics (8, 9).

Conversely, conventional histopathology quickly provides a wealth of irreplaceable data about structural integrity, spatial and temporal relationships, and rare events/cells. Only a tiny fraction of information is being harvested from tissue slides primarily because data extraction is dependent on a restricted staining repertoire and manual observation. The challenge, therefore, is to develop a modern replacement to the traditional histopathologic approach.

Dramatic advancements in robotics, digital imaging, and computing have spawned the “-omics” revolution that challenges routine histopathology for improved tissue utilization. Comprehensive examination of mRNA, protein, and metabolite expression data can be used as powerful screening tools to query disease susceptibility, pathophysiology, and prognosis. These same innovations, however, are also revolutionizing histopathology. High resolution whole slide image (WSI) scanners now enable pathologists to view routinely prepared and stained slides on a computer screen instead of a microscope. Pathologists can then team with hardware and software engineers, mathematicians, and image analysis experts to greatly increase the value equation for histopathology in an era when there is increasing pressure to diagnose and monitor liver diseases non-invasively(8).

We recently reported on the power of combining WSI, multiplex nanoparticle quantum dot staining, and automated image analysis to envisage and analyze multiple protein labels on a single slide to reveal biological mechanisms (7, 10–16). We use this novel approach to study liver epithelial diversity in formalin-fixed, paraffin-embedded, normal human liver tissue. In this report, automated quantitative data collection that described cell numbers, types, and nuclear/cytoplasmic analyte expression was spatially tethered to the tissue architecture. This enabled us to illustrate and locate pre-existing diversity within BEC and hepatocytes in normal human liver, including the transition zone between these cell types in the Canals of Hering. This might lead to a better understanding of the considerable diversity that quickly appears during disease states (5).

EXPERIMENTAL PROCEDURES

Tissue Sources and Multiplex Quantum Dot Immunolabeling

Four- μm sections from eight formalin-fixed paraffin-embedded and one frozen liver tissues were used for the analyses (Supplemental Table 1; Institutional Review Board protocol 0404010; University of Pittsburgh). Before study inclusion, H&E slides from each case were reviewed. Tissues were prepared and stained as previously described (7, 10). Liver and putative hepatic progenitor cell (HPC) or Canal of Hering (CH) characterization used the following panels (see Supplemental Table 2 for antibody listing): panel A [β -catenin (β -cat), cytokeratin 19 (CK19), α -smooth muscle actin (α SMA), and CD31]; and panel B [CK19, hepatocytes nuclear factor (HNF) 1 β , and HNF4 α].

High Resolution Whole Slide Scanning and Image Analysis

Slides were imaged with Mirax MIDI WSI scanner equipped with a Plan-Apochromat 40 $\times$ /95N.A. objective lens, AxioCam MRm digital CCD camera (Carl Zeiss, Jena, Germany) and specifically selected excitation/emission Qdot filters (Omega Optical, Battleboro, VT) as described (7, 10). Additional supporting three-dimensional wide field imagery was created using a robotic AxioImager M1 microscope (Carl Zeiss, Gottingen, Germany) equipped with various dry and oil-immersion high NA objectives and ZEN imaging software (Carl Zeiss, Jena, Germany) for microscope control and image visualization.

Pixel-based image analytics was performed using ImageJ (<http://rsbweb.nih.gov/ij/>); tissue-tethered cytometry analysis utilized FARSIGHT (http://www.farsight-toolkit.org/wiki/FARSIGHT_Toolkit) and IAE-NearCYTE (<http://nearcyte.org>). The tissue-tethered cytometry software packages are designed to delineate all cells, define cell types by user classifications, and quantify analyte expression. A total of 20 regions of interest (ROIs) were randomly chosen that centered on portal tracts or central veins (Supplemental Figure 1A). Each ROI was exported into individual grayscale JPGs without compression and imported into FARSIGHT tissue cytometry (7, 10) and ImageJ 1.45s for pixel analysis. Cell data obtained from FARSIGHT using panel A (β -cat/CK19/ α SMA/CD31/DAPI) staining was classified, analyzed and sorted by utilizing an active training system (candidates are user selected) based on nuclear size, elongation, CK19 intensity, β -catenin intensity, β -catenin total signal, CD31 intensity, and α SMA intensity. For certain expression patterns, data was selected and exported into a common delineated format for review with Microsoft® Excel®. (e.g. CK19=IF(CK19>1000, 1, 0), β -cat=IF(AND(nucleus size>23.4 μm^2 , β -cat total>15000, β -cat surrounding>3, CK19=negative, CD31=negative, α SMA=negative), 1, 0), CD31=IF(AND(CD31 average>48, CD31 surrounding>3, Nucleus size<57.2 μm^2 , CK19=negative, α SMA=negative), 1, 0), α SMA=IF(AND(α SMA average>38, CD31=negative), 1, 0). 1 = positive, 0 = negative. IAE-NearCYTE provides both pixel and cytometry features and was used to localize double and triple positivity on panel B (CK19/HNF1 β /HNF4 α /DAPI)-stained WSIs. Thresholds for fluorophore positivity were set manually and visual conformation was done after sorting to ensure specificity.

Statistical analysis

The statistical analysis methods to determine data sensitivity and significance were the t-test, Mann-Whitney U test, and one-way ANOVA test all performed using Sigma Stat version 11.0 (Aspire Software International, Ashburn, VA).

RESULTS

Comparison of Conventional Tissue Morphometry to Tissue-tethered Cytometry

Conventional image analysis of liver tissues (e.g. ImageJ, IAE-NearCYTE) has relied primarily on pixel-based determinations of *percent tissue area* occupied by a single analyte, channel, or stain assayed on individually-captured microscopic fields (Supplemental Figures 1 and 2). Examples include trichrome for fibrosis (9) or absence of staining for fat (17). Pixel-based analyses are powerful, but unable to easily provide information about cell-specific physical characteristics (size, shape, location) or complex data from multiple analytes, or social interactions. Common open source software (e.g. ImageJ) is rich in functionality for routinely captured static images but does not easily accommodate WSI.

Cell-based image analysis (e.g. FARSIGHT and IAE-NearCYTE) is a higher level image analysis approach based on grouping of similarly-colored pixels into biologically meaningful structures, such as cells and/or parts thereof. Each nucleus can serve as the nidus for cell-associated nuclear and/or cytoplasmic analyte(-s) (protein, DNA, mRNA) assays (Supplemental Figure 3). This enables identification of complex specific cell types based on Boolean logic relationships among multiple characteristics. For example, hepatocytes can be identified as cells with a relatively large ($> 23 \mu\text{m}^2$) round nucleus surrounded by β -catenin staining within a distance of $10 \mu\text{m}$ from the nucleus and negative CK19 staining (i.e. β -catenin^{far}/CK19⁻), whereas BEC are defined as smaller CK19⁺ cells. Cell-based approaches also enable the collection of data regarding location (X,Y) for two dimensional thin sections and Z planar addresses for thick sections, nuclear and cytoplasmic physical attributes (e.g. size, shape, and orientation characteristics), and nuclear and/or cytoplasmic analyte expression. Data collection can be followed by more sophisticated queries of social relationship.

Cell-based approaches also enable “tissue-tethered cytometry”. This refers to an ability to “virtually digest” the WSI. Each cell, regardless of size, shape, location, or phenotypic complexity, can be isolated and displayed in various formats. Examples include traditional and multi-dimensional scatter plots, whisker plots, and signaling pathway schemes derived from covariance relationships (data not shown). Importantly, individual cells in either scatter plots or WSI are tethered to the exact same cell on the complementary display. The observer can easily transition between displays to assess the cell from informational perspectives.

To distinguish between the two approaches, ten portal tracts and ten perivenular ROIs were selected randomly from panel A (CK19/ β -catenin/CD31/ α SMA/DAPI)-stained high resolution (40X) WSI images to determine the relative proportions of cell types in two separate livers (Supplemental Table 1, Supplemental Figures 1A–1B). As expected, α SMA⁺ cells were over-represented and BEC were found only in portal/periportal ROI compared to perivenular regions. FARSIGHT-generated data for hepatocytes, BEC, endothelial cell (EC), and smooth muscle cell (SMC) (Figure 1A) sorted from one liver (total 20 ROIs) yielded 539/18875 (2.86%) BECs; 9153/18875 (48.5%) hepatocytes; 1093/18875 (5.79%) EC; 669/18875 (3.54%) SMC, and 7421/18875 (39%) unclassified cells (e.g. nerve cells, leukocytes, quiescent stellate cells, etc). These results compared favorably with previously reported studies using tissue digestion and point-counting methods conducted on normal murine and human livers (18–22). Although difficult to compare, the current method is probably more sensitive and accurate because the WSI were created with a high resolution objective (Supplemental Figure 1B) and neither tissue disruption nor digestion, which can destroy and/or exclude cells, is needed. Since tissue-tethered cytometry enables harvesting of complex quantitative cell-specific data it was selected for all remaining analyses.

Normal Human Liver Tissue Cytometry

Quantitative cytometric data generated from tissue-tethered cytometry on various cell populations from normal adult livers confirmed previous observations using more laborious techniques (Supplemental Table 1 and Figure 1). For example, hepatocyte nuclei are significantly larger (Figure 1B) than all other liver cell types and perivenular hepatocyte nuclei are larger and more often binucleate ($7.7 \pm 1.8\%$ vs $6.4 \pm 1.0\%$; $p < 0.001$; Mann-Whitney t-test, Figure 1D) than periportal hepatocytes (Figure 1B) (reviewed in (23)). BEC nuclei lining large septal bile ducts ($80 - 150 \mu\text{m}$ diameter) were also significantly larger than BEC nuclei lining small bile ducts from the same liver ($< 25 \mu\text{m}$ diameter; Figure 1C), as previously shown in rodent whole liver digestion studies (24).

The record of individual cell X-Y coordinates enabled a diagrammatic reconstruction of histological structure (Figure 1D), which can be used for: 1) quick visual inspection quality control of cell identification and sorting characteristics; 2) social relationship between cell types; and 3) easy identification of specific cell types or rare events/cells within the context of tissue structure. For example, the number of nearest neighbors at predetermined radii from each hepatocyte (Figure 1E left and right upper, yellow lines) can be easily calculated. Based on a training set optimal distance of $35 \mu\text{m}$, nearest neighbor calculation showed that hepatocytes with larger nuclei ($> 100 \mu\text{m}^2$) showed fewer nearest neighbors (more widely separated) than hepatocytes with smaller nuclei ($< 100 \mu\text{m}^2$; Figure 1E; 4.7 ± 2.1 vs 5.5 ± 2.2 ; $p < 0.001$; Mann-Whitney t-test). Since hepatocyte nuclear and cytoplasmic sizes are directly proportional, more distant neighbors for larger nuclei are expected. Clustering of smaller cells, however, is not widely appreciated. This could be related either to polarization of nuclei to nearby edges of cells or small cells, or both. Studies are underway to further investigate this finding.

BEC Analysis

Reassured that software-generated data on WSI reproduced previously accepted and verified results for hepatocyte and BEC sizes and their distribution, we more closely examined CK19+ BEC using CK19/ β -cat/ α SMA/CD31-stained WSI. Scatter plots generated from portal/periportal-based ROI consistently showed a population of CK19^{weak} cells. Taking advantage of “tissue-tethered cytometry”, we queried the location of these CK19^{weak} cells; a majority was located immediately adjacent to periportal hepatocytes. These characteristics correspond to accepted Canal of Hering (CH) definitions (25, 26), which are thought to contain putative hepatic progenitor cells (HPC).

We next manually selected 10 portal/periportal regions of interest (ROI), harvested cytologic characteristics of all software screened and human verified CK19^{weak} cells that fulfilled location characteristics of CH cells. A total of 12 putative CH cells fulfilled these characteristics. These software-screened and human-selected CH cells or putative HPC characteristics were compared to otherwise typical mature BEC lining portal tract bile ducts and midzonal hepatocytes. CH cells showed significantly lower CK19 expression (CK19^{low}; Figure 2A) and identical β -catenin expression (β -catenin^{close}; Figure 2B) compared to typical mature BEC lining portal tract bile ducts. Nuclear size, however, was intermediate between typical BEC and hepatocytes (Figure 2C). CH cells also had a very high nuclear:cytoplasmic (N:C) ratio and a close relationship to CD31+ sinusoidal EC (Figure 2D).

If the software-generated phenotypic characterization of CH cells is accurate, we should be able to use this technique and identify “rare event” CH cells in new image sets based on training set characteristics. Results from a representative experiment are shown in Figures 2E–H. CK19^{low} candidate cells were first gated (Figure 2E) and then sorted according to

their boundary profile (0=rare touching neighbors; 0.07=more touching neighbors similar to typical BEC lining bile ducts) and β -catenin intensity (Figure 2F). Visual inspection of software-identified putative HPC in new tissue sections confirmed localization to CH (green arrows in Figure 2G and high magnification in Figure 2H). In contrast, high boundary=0.07 CK19⁺ cells were located within otherwise typical bile ducts (red arrowheads in Figure 2G). The same analysis of three separate livers using CK19^{low} as the only discriminating criterion yielded a specificity 0.72 ± 0.13 for software-selected CH cells when compared to human identification which is CK19⁺ cells in periportal parenchyma adjacent to hepatocytes (25). When low boundary scores were combined with the CK19^{low} criterion, the specificity for software-selected CH compared to human increased to 0.92 ± 0.02 (t-test, $p=0.046$).

We next examined CH cells acquired in multi-focal planar wide field images using panel A-stained (CK19/ β -cat/CD31/ α SMA/DAPI) 20- μ m-thick sections to further examine CK19 expression in CH cells (Figure 3). The multi-focal imagery was created using a 100X objective lens scan on an AxioImager M1 microscope (Carl Zeiss, Gottingen, Germany) equipped with software control for autofocus and field stitching to create a seamless wide field representation. The resultant image stack was processed via deconvolution methods using Zen software (Carl Zeiss, Munich, Germany) to render a 3-dimensional composite (Rotation in 3-dimensions can be viewed at: <http://youtu.be/YGbyy9WoXz8>). CH CK19^{low} cells (* in Figure 3) showed a cuboidal shape and weaker CK19 expression (white arrowhead in lower left panel) at the cell border immediately adjacent to the hepatocyte (white arrowheads in Figure 3). Serial virtual step sectioning is shown Supplemental Figure 4 (Digital movie examples of the CH-Hepatocyte junction at 40X and 100X magnification can be seen at: (40X) <http://youtu.be/JWyz4h9IUvk>; (100X) <http://youtu.be/ww99LiX0N0E>).

Transcription Factor Expression in Liver Epithelial Cells

Hepatocyte-associated nuclear transcription factor HNF4 α (27) and biliary associated transcription factor HNF1 β (28, 29) are essential for development and phenotypic maintenance of hepatocytes and mature BEC, respectively. We next determined whether any hybrid or bipotential (HNF1 β ⁺/HNF4 α ⁺) cells existed in bile ducts and periportal regions of normal adult human livers. Portal/periportal ROIs from five normal human livers were subjected to analysis and results displayed on multi-parameter scatter plots showing nuclear size and signal intensity for CK19, HNF1 β , HNF4 α (Figure 4A). Tissue-tethered cytometry showed that most CK19⁺/HNF1 β ^{strong}/HNF4 α -cells with small nuclei mapped back to otherwise typical BEC lining portal tract bile ducts (cells 'd' in Figure 4B), as expected. Most CK19⁻/HNF1 β ⁻/HNF4 α ^{strong} cells with large nuclei localized to otherwise typical mature hepatocytes (cells 'e' in Figure 4B), as expected.

Two distinct populations of HNF4 α ⁺/HNF1 β ⁺ cells were identifiable in the X=HNF4 α , Y=HNF1 β scatter plot (Figure 4A): 1) HNF1 β ^{strong}/HNF4 α ^{weak} with a small nucleus (BEC-type) that showed phenotypic characteristics of BEC on routine light microscopy and 2) HNF1 β ^{weak}/HNF4 α ^{strong} with a larger nucleus (hepatocyte-type) that showed phenotypic characteristics of hepatocytes on routine light microscopy. Transcription factor localization was verified using single immunoperoxidase labeling of frozen sections of normal human livers (Supplemental Figure 5). The observation that the quantitatively dominant transcription factor controlled the routine light microscopic phenotype of cells substantiated the validity of this approach: HNF4 α -dominant cells appeared as hepatocytes and HNF1 β -dominant cells appeared as BEC.

Tissue tethering was used to localize the various HNF1 β ⁺/HNF4 α ⁺ populations. CK19⁺/HNF1 β ^{high}/HNF4 α ^{low} BEC-type cells (white cells 'a' in Figure 4B) localized to CH, but were rare. HNF1 β ^{low}/HNF4 α ^{high} hepatocyte-type cells (yellow cells 'b and c' in Figure 4B)

localized to the interface zone of normal livers and two different morphological subtypes of CK19-/HNF1 β ^{weak}/HNF4 α ^{strong} hepatocyte type cells were observed among periportal hepatocytes: one showed an oval-shaped intermediate-sized nucleus ('b' in figure 4B); the other contained a large round typical hepatocyte nucleus ('c' in figure 4B). Among the various cell types at the interface zone, the rarest cell was CK19+/HNF1 β -/HNF4 α + cell (Navigation of the WSI using toggle switches to turn off/on various analytes can be viewed at: <http://youtu.be/WldIOIzfQVM>).

Various HNF1 β +/HNF4 α + cell types showed phenotypic and analyte expression characteristics intermediate between otherwise typical BEC lining portal bile ducts and hepatocytes (Figure 4C). For example, HNF1 β ^{high}/HNF4 α ^{low} BEC-type cells showed lower CK19 expression than mature BECs, as expected. CK19-/HNF1 β ^{weak}/HNF4 α ^{strong} hepatocyte-type cells expressed significantly higher HNF1 β than mature hepatocytes and significantly higher HNF4 α expression than mature BEC (Figure 4C). Cells most strongly positive for both HNF1 β + and HNF4 α + often showed hepatocyte features with an oval nucleus, but lacked CK19 staining and were close to CH cells or terminal bile ducts, but a direct connection could not always be confirmed in thick sections (Figure 4D). A graphic reconstruction summarizing our results is shown in Figure 5.

DISCUSSION

HNF1 β (28, 29) and HNF4 α (reviewed in (27)) are responsible for development and maintenance of mature BEC and hepatocyte phenotypes, respectively. In agreement with previous single marker studies, HNF1 β can be expressed by periportal hepatocytes (28, 29) and HNF4 α can be expressed by occasional BEC (30). A novel workflow with multiplex labeling, however, enabled us to show that the quantitatively dominant transcription most strongly influenced the routine histopathologic appearance of the cells. Indeed, multiplex labeling, WSI creation, and automated image analysis enabled us to identify and characterize diverse epithelial populations that show transitional cytometric characteristics and analyte expression, including co-expression of HNF1 β and HNF4 α : 1) CK19+/HNF1 β ^{high}/HNF4 α ^{low} BEC-type cells; 2) CK19^{weak}/HNF1 β ^{high}/HNF4 α ^{low} BEC/CH-type cells; 3) CK19-/HNF1 β ^{weak}/HNF4 α ^{strong} hepatocyte-type cells with an oval nucleus; and 4) CK19-/HNF1 β ^{weak}/HNF4 α ^{strong} hepatocyte-type cells with a large round nucleus (identical to mature hepatocytes). This extensive, but difficult to visualize with conventional histology, population of cells with phenotypic and biomarker (including transcription factor) expressions of a hybrid nature between hepatocytes and biliary epithelial cells are positioned over a relatively broad area from small portal-based bile ducts to otherwise typical periportal hepatocytes.

Previous studies in rodents show that progenitor cells (i.e. "oval" cells) arise from BEC to provide hepatocytes when regeneration of the liver needs to occur under conditions in which hepatocyte proliferation is inhibited(31). Similar conclusions have been reached in cirrhotic human liver tissue samples where hepatocytes are thought to be derived by terminal bile ducts/Hering Canals harboring putative progenitor cells(32–34). Conversely, periportal hepatocytes can give rise to BEC when BEC are incapable of regenerating (35). The phenotypic transitions between hepatic epithelial cells, or hybrid hepatobiliary cells, which are experimentally well-characterized in rodent liver biology, also occur in human liver during ductular reactions (1–3, 5, 26, 36–38) and when otherwise typical hepatocytes appear intermixed among BEC in portal tract bile ducts(4).

Location of these hybrid transitional cells in portal bile ducts, canals of Hering and immediate periportal hepatocytes of normal liver coincides with the niche of potential stem cells described in rodent liver(39). Like their studies, we cannot exclude the possibility that

bipotential periportal HNF1 β + / HNF4 α + hepatocytes are derived from Hering Canal cells(39) given their often close proximity (see Figure 4). However, given the pre-existence of such hybrid cells in the normal liver and abundance of hybrid epithelial phenotypes in diseased human livers, we postulate that the former expand to give rise to the latter when the liver microenvironment is disrupted. It is likely that latent hybrid cells play an important role during extreme regenerative situations when a need exists for facultative stem cells to rescue the regenerative failure of one or the other liver epithelial cell compartments. As such, they are likely to play an important role in understanding the pathogenesis of human liver disease.

Use of digital imaging and computational image analysis in liver pathology, to date, has been largely limited to capture of single microscopic fields followed by pixel-based determination of fat quantities(17) and fibrosis areas(9). This approach, however, has a low value proposition: it is impractical and time-consuming for marginal improvements in information extraction (9). Multiplex labeling techniques to identify complex cell phenotypes that also express target proteins of interest are also impractical, expensive, and inconvenient with traditional imaging methods(40) because of: a) reliance on traditional fluorophores with inherent drawbacks; b) expensive and inconvenient fluorescent microscopes; and c) dependence on tedious image capturing steps and subjective interpretation. Spatially overlapping signals derived from brightfield chromogenic multiplexing are harder to separate and quantify unless multispectral imaging is used (41).

In this study, we introduced a workflow that combines high resolution digital imaging, robotics, computing, nanotechnology, and software “toolkits” that enable pathologists and researchers to extract more biologically significant cellular information from tissue samples than is currently achieved by human analysis alone. “Tissue cytometry” was first described nearly ten years ago (42), but was only recently applied for tissue analysis, spurred by convergent advances in computer processing, imaging equipment, and staining materials/protocols that made the overall process more practical (reviewed in (7, 43)).

Particular strengths of tissue-tethered cytometry include ability to: a) collect detailed quantitative physical (e.g. nuclear size, location, etc.) and analyte (protein, DNA, RNA) data on thousands of cells; b) “virtually digest” the tissue while retaining structural context; and c) represent the data in a variety of formats (e.g. multi-dimensional scatter plots, diagrams, signaling pathways, heat maps, and clustering diagrams) that might be more easy to interpret than the image itself or in conjunction with the image; d) map social relationships among cells; and e) identify or screen for rare event candidates followed by human quality assurance review (e.g. putative HPC; Figures 2 and 4).

The workflow technology described herein is being routinely applied for basic science and clinical trial research purposes (7, 10–16), but limitations exist for routine clinical implementation: 1) serial, and time consuming, nature of the staining; 2) uneven tissue staining; 3) long scan time required for creation of multiplex digital images; 4) large amount of data generated; and 5) the need to train pathologists. Automatic nucleus segmentation is still a challenge when cell nuclei overlap in thick or inflamed tissue sections or when DAPI signal intensity varies across hepatocytes and stromal cells. While common methods for nuclear segmentation histogram-based, clustering-based, and entropy-based algorithms (44) are often used, recent higher specificity model based computational methods are becoming practical because of improved computational power. Software interfaces to WSI are not yet platform agnostic, as such hybrid methods using multiple tools still require specialty informatics techniques to be used to repackage and import imagery data into the various programs(44).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

β-catenin	beta-catenin
BEC	biliary epithelial cell
CD31	cluster of differentiation 31
CH	Canal of Hering
EC	endothelial cell
HNF	hepatocyte nuclear factor
HPC	hepatic progenitor cell
ROI	region of interest
WSI	whole slide image
SMA	smooth muscle actin
SMC	smooth muscle cell

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Figure 1. FARSIGHT image overlay on CK 19 (green)/β-catenin (cyan)/CD31(red)/αSMA(yellow)/DAPI(blue) panel staining on normal adult human liver (See Supplemental Table 1) illustrates the process of individual cell classification. (A) Each nucleus is assigned an ID number and XY coordinates marked by a centroid placed at the geographical center of each nucleus. Nuclei are outlined by a binary marker (red outline) to identify each nucleus as negative (cyan) or positive (red) for the marker(-s) of interest. Each cell can then be sorted or displayed according to nuclear size, segmentation, and signal intensity and identified as hepatocytes, biliary epithelial cells (BEC), CD31+ endothelial cells, and αSMA+ smooth muscle cells. (B) Hepatocytes from perivenular areas (hepatocytes(c)) have significantly larger nuclei than periportal hepatocytes (hepatocytes(p)) and other cell types. (ANOVA; $p < 0.05$, *: $p < 0.01$). The same trend was observed in another liver. (C) BEC nuclei from small bile ducts (< 25 μm diameter) are significantly smaller than BEC lining septal (80 –

150 μm diameter) bile ducts (BD) from the same liver (t-test, $p < 0.001$). The image shows representative BECs from each anatomical level with (lower panels) or without (upper panels) CK19 (green) signal. This result was reproducible in another normal liver. (D) Cell-based data can be used to reconstruct the original image as a diagram (lower panel), which is nearly identical to the actual image (upper panel), validating the process for deconvolution of CK19 (green circles)/ β -catenin (cyan squares)/CD31 (red circles)/ α -SMA (yellow triangles). Specific information, such as binucleate hepatocytes (insert in original upper image), can be displayed as distinct symbols (red trim cyan squares) (see legend). (E) Nearest neighbor calculations on a portal-based ROI (upper left panel; merged) within training set defined distance (35 μm) reveals a network of connections as yellow lines (upper right panel; Neighbors network). Colored-coded rendering of hepatocyte nuclear size (lower left panel; Rendering of nucleus size) and number of nearest neighbors (lower right panel; Rendering of number of neighbors; see key on figure) can be used to show that hepatocytes with large nuclei also have more cytoplasm, and thus, fewer close neighbors. Hepatocytes with smaller nuclei have more neighbors and tend to form small clusters. Identical results were obtained in another adult male liver.

Figure 2.

Canals of Hering (CH) in human normal liver tissue in CK19(green)/β-catenin(cyan)/CD31(red)/αSMA(yellow)/DAPI(blue)-panel stained WSI. CH cells selected as described in the text, were immediately adjacent to hepatocytes and showed: (A) lower CK19 intensity; and (B) β-catenin expression similar to mature BECs lining bile ducts within portal tracts, but higher than in hepatocytes. (C) Nuclear size of BEC lining small bile ducts (80 – 150 μm diameter) was significantly lower than hepatocytes (ANOVA, $p < 0.001$), as shown in Figure 1, whereas CH cell mean nuclear size was intermediate between otherwise typical BEC and hepatocytes. (D) Example of CK19^{weak}(green)/β-catenin+ (cyan) CH cell (white arrow) was found in the subsinusoidal space, isolated from portal-tract-based bile ducts and immediately adjacent to a small hepatocyte and CD31+ sinusoidal endothelial cell. (E) CK19^{weak} cells are gated, and (F) sorted according to the ‘nuclear boundary’ score and β-catenin. Boundary=0 cells (green gate) are CH cell candidates whereas boundary=0.07 cells (red gate) are portal bile duct BECs. (G) Green arrows highlight cells from the green gate in (F) showing that they localize near the edge or slightly outside of portal tracts. In contrast, cells from the red gate in (F) localize to BEC lining portal tract bile ducts. (H) High magnification (1000X) of CH cells showing distinct β-catenin expression, but CK19 staining was weak and incompletely surrounded this cell, which was adjacent to a hepatocyte. The fine red linear circle outlining the nucleus indicates a “software selected” object nucleus, which fulfills pre-determined analyte characteristics used to define this cell population.

Figure 3.

Three-dimensional view of panel A multiplex [CK19(green)/ β -catenin(cyan)/CD31(red)/ α SMA(yellow)/DAPI(blue)] staining on 20 μ m section of normal 50 year old male human liver. The upper left panel shows a depth rendering where the red color highlights edges closest to the front face of the cube (level 0 μ m), whereas the blue color is expressed on the opposite face (level 3.5 μ m). Thus, most of the β -catenin staining is located at the edge face of the hepatocyte of interest. Shown is a CK19^{low} (lower left and lower right panels; green; * terminal bile duct) cell, or Hering Canal cell, that attaches directly to a mature hepatocyte (upper and lower right panels; cyan; three white arrowheads points to junction). The junction between the two cells is highlighted by the white arrowheads (► from BEC side, and ◄◄◄ from Hepatocyte side). Note the weak CK19 staining on the side of the CH cell immediately adjacent to the hepatocyte (►).

Figure 4.

(A) Representative multi-dimensional scatter plots using data extracted from panel B-stained slides [CK19(cyan)/HNF1β(green)/HNF4α(red)/DAPI(blue)]. 3D plot (X=CK19, Y=HNF1β, Z=HNF4α, blue to red represents nuclear size from small to large) shows some specific clusters. CK19⁺ cells have small nuclei, as expected. CK19 vs HNF4α shows the vast majority (~99%) of HNF4α⁺ cells are CK19⁻, as expected. The separate HNF4α vs HNF1β plot shows that 0.26% of all cells were HNF4α⁺/HNF1β⁺ (double positive (DP)) and two populations are observed. HNF4α^{strong}/HNF1β^{weak} DP (hepatocytes-type) cells are significantly larger (red dots in scatter plots) than HNF4α^{weak}/HNF1β^{strong} DP (BEC-type) cells (see text). (B) Example of cell classification overlay on panel B [CK19(cyan)/HNF1β(green)/HNF4α(red)/DAPI(blue)] 4μm WSI normal adult human liver. Object masks (nuclear overlay) classify cells into single positive for HNF1β (red), HNF4α (green), and CK19⁺ (cyan). HNF1β⁺/HNF4α⁺ DP cells are yellow, CK19⁺/HNF1β⁺/HNF4α⁺ cells are white, and CK19⁺/HNF1β⁺ cells are pink. Several different types of periportal HNF1β⁺/HNF4α⁺ DP cells are detected. Quantitative data are displayed in the Table at the lower right corner panel of the screen shot image. “Double clicking” on any cell with a nuclear overlay highlights corresponding quantitative data in the Table. Representative examples for various cell types are shown in the right panel. a: HNF1β^{high}/HNF4α^{low}/CK19^{weak} BEC-type, b: HNF1β^{low}/HNF4α^{high}/CK19⁻ hepatocyte-type cell with an oval-shaped nucleus, c: HNF1β^{low}/HNF4α^{high}/CK19⁻ hepatocyte-type with a large, round, otherwise typical,

hepatocyte phenotype, d: HNF1 β ⁺/HNF4 α ⁻/CK19⁺ mature BEC, e: HNF1 β ⁻/HNF4 α ⁺/CK19⁻ mature hepatocyte. (C) Comparison of cell characteristics pooling data from 5 separate normal adult livers showed that HNF1 β ^{high}/HNF4 α ^{low}/CK19⁺ BEC-type cells tend to have slightly larger nuclei than mature BECs. HNF1 β ^{low}/HNF4 α ^{high} hepatocyte-type cells showed larger nuclei than mature BECs, as expected. HNF1 β ^{high}/HNF4 α ^{low}/CK19⁺ BEC-type DP cells express CK19 at a lower intensity than mature BEC and significantly higher HNF1 β expression than mature hepatocytes. HNF1 β ^{low}/HNF4 α ^{high} hepatocytes-type cells had significantly higher HNF4 α expression than mature BECs. (D) Panel B staining on 20 μ m section of normal 50 year old adult human liver. The horizontal slice (green frame) and vertical slice (red frame) are shown on the left and top side of each panel, respectively. A HNF1 β ⁺/HNF4 α ⁺ (yellow) cell is immediately adjacent to CK19⁺ CH cells (cyan), but CK19 is not expressed by this HNF1 β ⁺/HNF4 α ⁺ (yellow) cell that shows an oval nucleus, relatively abundant cytoplasm and relatively large nucleus similar to otherwise typical hepatocytes.

Figure 5.

Diagrammatic representation of the connection between hepatocytes and bile ducts through the Canals of Hering based on tissue-tethered cytometric analysis. Portal bile ducts form small channels lined by 3–4 BECs that traverse the limiting plate into the periportal lobule, which ends, often in a single layer, directly attached to periportal hepatocytes. Triangular-shaped cells in CH have slightly lower CK19 expression than mature BEC often on the face bordering the HNF1β^{low}/HNF4α^{high} hepatocytes with typical large round or oval-shaped nuclei. The letters a, b, and c, correspond to the letters used to identify various HNF1β⁺/HNF4α⁺ populations identified in Figure 4. PV: portal vein, BD: bile duct, HA: hepatic artery, EC: endothelial cell, HSC: hepatic stellate cell. The nuclear color code is provided at the bottom of the image.