Profiling immune cells in the kidney using tissue cytometry and machine learning

Seth Winfree¹, Mohammad Al Hasan² and Tarek M. El-Achkar¹,³

1- Department of Medicine Division of Nephrology, Indiana University, Indianapolis

2- Department of Computer Science, Indiana University-Purdue University, Indianapolis

3- Indianapolis VA Medical Center, Indianapolis

Correspondence:

Tarek M. El-Achkar (Ashkar), MD
950 W Walnut St, R2-202
Division of Nephrology
Indiana University
Indianapolis, 46202
Email: telachka@iu.edu
Abstract

The immune system governs key functions that maintain renal homeostasis through various effector cells that reside in or infiltrate the kidney. These immune cells play an important role in shaping adaptive or maladaptive responses to local or systemic stress and injury. We increasingly recognize that microenvironments within the kidney are characterized by unique distribution of immune cells, the function of which depends on this unique spatial localization. Therefore, quantitative profiling of immune cells in intact kidney tissue becomes essential, particularly at a scale and resolution that allow the detection of differences between the various “nephro-ecosystems” in health and disease. In this review, we discuss advancements in tissue cytometry of the kidney, performed through multiplexed confocal imaging and analysis using the Volumetric tissue exploration and analysis (VTEA) software. We highlight how this tool has improved our understanding of the role of the immune system in the kidney and its relevance in pathobiology of renal disease. We also discuss how the field is increasingly incorporating machine learning to enhance the analytical potential of the imaging data and provide unbiased methods to explore and visualize multidimensional data. Such novel analytical methods could be particularly relevant when applied to profiling immune cells. Furthermore, machine learning approaches applied to cytometry could present venues for non-exhaustive exploration and classifications of cells from existing data and improving tissue economy. Therefore, tissue cytometry is transforming what used to be a qualitative assessment of the kidney into a highly quantitative imaging-based “omics” assessment that compliments other advanced molecular interrogation technologies.
Importance of the immune system in the kidney

The human kidney comprises at least 18 types of cells that are organized spatially in unique structures and zones within the kidney (1). This unique distribution allows the performance of specialized physiological functions and shape the response to disease (2). Cells of immune system are distributed throughout the renal interstitium and are in intimate cross-talk with other cell types such as the tubular epithelium (3-6). Immune cells are key regulators of the renal milieu in homeostatic conditions (5-8). They comprise resident phagocytes such as macrophages and dendritic cells, but also occasional “visiting cells”, such as neutrophils and lymphocytes that may marginate and cross over from the microcirculation in response to local signals (5, 9, 10). Example of homeostatic functions include immune monitoring and tolerance to non-harmful antigens and maintaining infection defense capability (5, 6, 8, 11).

In disease, systemic and local events alter the composition, phenotype and function of resident cells (6, 12, 13). For example, activated antigen presenting cells travel to regional lymph nodes (14). Resident dendritic cells and macrophages activate gene expression programs driven by cues from other cells and the environment (15). In addition, chemokines release by injured or stressed cells will attract more cells from the circulation, such as neutrophils, monocytes, natural killer cells and lymphocytes (16, 17). Upon entering the kidney milieu, many cells undergo phenotype switching (18). For instance, responding to environmental cues, monocyte transform into specific macrophage phenotypes. Many of these cells could also start proliferating within the kidney, providing additional “clones for the war” (12, 19). The dynamics and fate of these infiltrating cells will depend on the course and severity of disease.
In an adaptive response, the disease is controlled, and the function of immune cells changes from danger mitigation to promotion of healing (18, 20). Such transformation requires curtailing cells that could become dangerous to self, if left unchecked, such as neutrophils which are directed to die or leave the kidney (21). Furthermore, macrophages shift to a healing phenotype, by removing dead cells, debris, and secreting anti-inflammatory signals (20, 22, 23). Non-immune cells can then regenerate and restore other functions (24). In a maladaptive response, which could result from a persistent or very severe injury, persistently activated immune cells can cause damage to bystanders and perpetuate the injury (18, 22, 25). The result could be a migrating pattern of injury, leaving behind fibrosis and on-going damage. Such a model is thought to be a key pathogenic feature of chronic disease and in progression from acute to chronic disease. Therefore, the immune system is a key regulator of kidney health and disease.

**Available methods to assess immune cells within the kidney and the importance of spatial information**

Technologies to quantitatively survey the type of immune cells and their changes within the kidney have experienced revolutionary growth over the last decade. The application of flow cytometry, transcriptomics and more recently single-cell transcriptomics of cells from homogenized kidney tissues have provided important advancements to our understanding of the role of the immune system in the kidney (5, 12, 13, 26-29). The identification of profiles of resident and infiltrating immune cells in health and disease based on specific markers have been crucial for progress (5, 6, 12, 15, 29). Moreover,
the importance of spatial distribution in determining various functions of renal immune cells within the kidney is increasingly appreciated (5, 7, 8, 11, 18, 30). As we understand the impact of the microenvironment on the pathogenesis of injury and the interactions between various cell types, it becomes imperative to qualify and quantify immune cells and their interactions with the various “ecosystems” within the kidney. This fits with what we know about the physiological function of the kidney, where specialized tasks are performed in a specific spatial context. For example, Berry et al showed that renal hypertonicity dictates the localization and function of a specific set of mononuclear phagocytes, the function of which is needed for antibacterial defense from ascending infection (11). Hochheiser et al showed that cortical and medullary dendritic cells play specialized role in their renal microenvironment (30). Based on surface marker expression, we previously showed that the distribution of specific sets of mononuclear phagocytes is also specific to renal zones. For example, resident macrophages defined as CD45+, LY6G-, F4/80 hi, CD11B low are more abundant in the medulla than cortex (31). The importance of the spatial distribution of immune cells is particularly relevant in disease pathogenesis and course. Ischemia-reperfusion predominantly involves the outer medulla, where neutrophils and other phagocytes infiltrate shortly after the onset of injury. However, with chronic kidney disease, active inflammation and fibrosis are frequently detected in the cortex (32). In experimental glomerulonephritis, a subset of key pathogenic dendritic cells are uniquely localized near blood vessels (33). Similarly, the renal microenvironment and epithelial immune interactions governs the site of antigen presentation in allograft rejection (7), and the association of infiltrating macrophages with specific tubules in sepsis (34). These are just few examples of the
importance of accurately identifying the spatial distribution and neighborhoods of resident and infiltrating immune cells in health and disease.

To perform such a task, tissue preserving strategies that survey immune cells \textit{in situ} are needed. This is performed by an imaging approach that is linked to an analytical pipeline that can process the imaging data through an intuitive and interactive interface, which can provide quantitative outputs. Many such approaches have been described and have advantages and limitations (35-50). We recently reviewed such approaches and would refer the reader to these previous works (51, 52). In the current review, we will focus on tissue cytometry of the kidney using large scale 3D confocal fluorescence imaging and tissue cytometry. Our group developed the Volumetric Tissue Exploration and Analysis (VTEA) software, a tool that could be used to analyze 3D image volumes from kidney tissue (51, 53). We will describe the workflow and application of 3D cytometry using VTEA to analyze the immune system within the kidney. We previously demonstrated the importance of imaging in 3D, particularly for immune cells, whereby the expression of surface markers used for identification can be unevenly distributed across the cell (53). Therefore, capturing entire cells and structures in 3D will enhance the accuracy of classification.

**Tissue cytometry description- VTEA workflow**

\textbf{Tissue preparation and imaging:} Large scale 3D imaging of kidney tissue can be obtained from frozen sections or from formalin fixed and vibratome-sectioned tissue. For abundant tissue sources such as experimental murine kidneys, we prefer vibratome
sections (54), since the morphology is exceptionally preserved by immediate fixation and avoidance of freezing. However, for sparse tissues such as human kidney biopsies, we frequently share the tissue with clinical applications and use optimal cutting temperature compound (OCT)-embedded frozen sections, since they are the most compatible with our imaging process (53, 55). Frozen specimen can be sectioned into 50 um-thick sections and immediately fixed with paraformaldehyde to prepare the tissue for staining. The detection of immune and other cells within the kidney depends on the availability of reliable markers and antibodies that produce rigorous and reproducible results (56). This is a major rate limiting step on the type of cells that can be detected by this technology (i.e. subpopulations and various subtypes). Although standard confocal microscopy can simultaneously detect up to 4 labels spread across the fluorescence spectrum without any significant bleed through between acquisition channels, it is possible to increase the number of simultaneously detected fluorophores up to 8 with spectral deconvolution (52, 55). To efficiently perform 3D tissue cytometry, a channel must be dedicated to stain the nuclei, which will serve as fiduciaries to survey all the cells. Large scale 3D imaging is performed by tile scanning and digital stitching of the acquired volume. Such an approach will assure mesoscale imaging at a subcellular resolution, a great strength of this technology (51-53). The process for staining and imaging has been standardized and is made available on protocols.io (dx.doi.org/10.17504/protocols.io.9avh2e6).

**Image analysis and cytometry:** VTEA was designed and built as a plugin for the commonly used ImageJ/FIJI tool for image analysis (51-53). VTEA is meant to be intuitive, where all the steps needed from visualization, image processing and analysis
can be done in a single space (**Figure 1**). The workflow of VTEA is bidirectional (between image processing and analysis), whereby the flexibility of its design allows for real-time adjustments of parameters in the pre-processing and segmentation to optimize the analysis. The premise of the analysis is that every nucleus can be segmented automatically using built in functions within VTEA and ImageJ. Each surveyed nucleus becomes a surrogate for its cell, to which the location (coordinates) and marker staining around or within the nucleus can be registered. This captured information can be classified as label-based (e.g. fluorescence mean intensity, intensity distribution, texture, etc.), spatially-based (e.g. X, Y, Z coordinates, neighborhoods, etc.), or combined (e.g. directionality of the intensity). These three classes of information become measured variables (features) associated with each nucleus/cell. The number of features associated with each cell can be expanded experimentally by applying multiple fluorescent labels (more primary variables) or computationally by inferring new features from existing labels and spatial distribution (more derived variables). Consequently, the feature space is potentially limitless. However, for a directed analysis, we tend to focus on specific features such as the mean intensity fluorescence of each marker associated with each cell (53, 55). The data (objects where each dot represents a cell) is plotted as a scatter plot where the X and Y axis each present a feature, and the color can be used to represent a third feature (**Figure 1D**). Cells of interest can then be identified by these features, and a gate drawn to perform quantitation. An important characteristic of VTEA is that it allows back-mapping of the analysis onto the image volumes, so that the cell of interest identified by a gate are directly visualized as colored overlays on the image, with the corresponding displayed
statistics (Figure 1D and E) (31, 51-53, 55). This functionality allows the immediate interpretation and validation of the gates. In addition, direct gating on the image can be performed, which can trace all the cells within the chosen region-of-interest back to the data display on the scatter plot (Figure 1F and G). Such a back-and-forth interplay between the image volumes and the quantitative plots allows the interactive exploration and analysis of the data.

Limitations of cytometry with VTEA: Although VTEA includes a suite of state-of-the-art tools for visualization, image processing and analysis, these tools may not be the best solution for all circumstances. We understood this limitation early in developing VTEA and have implemented an extensible framework. Therefore, VTEA can be dynamically extended with emerging and potentially more effective tools for visualization, image processing and analysis (52). Furthermore, the workflow is adaptable as segmentations and features may be imported from other software or workflows (e.g. CellProfiler and other FIJI plugins such as MorphoLibJ) (57, 58) for use in VTEA. These capabilities will be described in further detail in an upcoming publication.

Profiling the immune system with tissue cytometry in experimental mouse and human disease

We have applied tissue cytometry to survey immune cells in various experimental settings in animal models and human specimens. In mouse kidneys, we used VTEA to quantify the distribution of various subtypes of immune cells based on MHCII and CD11C staining and determined that the cortex and medulla have a differential
distribution of renal mononuclear phagocytes based on these two markers (53).
Furthermore, we determine that deficiency of uromodulin, a protein uniquely produced
by the thick ascending limbs and early distal tubules, causes a selective deficiency of
phagocytes in the inner stripe of the outer medulla (53). Furthermore, we used VTEA to
quantify the interaction between uromodulin and CD11C+ cells and showed that 13% of
CD11C+ cells also had evidence of uromodulin staining (31), suggesting significant
uptake of uromodulin within the interstitium by these phagocytes. This is of great
biological relevance because uromodulin is an important determinant of the number and
function of mononuclear phagocytes within the kidney (31). Using VTEA, we also
developed an in vivo quantitative phagocytosis assay by injecting fluorescent beads in
live mice and quantifying the uptake of beads by MHCII+ cells in specific renal regions
(31). Ongoing efforts are underway to characterize the spatial distribution of various
immune cells during various types of kidney injury (ischemic, sepsis, nephrotoxic) and
link the distribution of immune cells to injury signals from the subtypes of tubular
epithelium (Figure 2). Finally, we have also started applying cytometry to pathway
imaging, by labeling for activated proteins in the kidney (52). For example, we showed
that activation of p-c-Jun can be quantified in the kidney using tissue cytometry (59).
Such approach could be very useful in profiling the activity of immune cells in the kidney
during disease.

Large scale 3D imaging and tissue cytometry is also being used in quantifying immune
cell abundance and distribution in human kidney tissue, in health and disease (Figure
3). We previously showed that this approach could be used in archived frozen human
kidney biopsies, whereby quantitation of immune cell densities could be potentially linked with other molecular or clinical features (51, 53). Large scale 3D imaging and tissue cytometry of human kidney biopsy is currently being used as a main imaging technology in the Kidney Precision Medicine Project (KPMP) consortium (55, 56, 60). This NIDDK supported efforts is one of the largest consortia studying the pathogenesis of human kidney disease by safely and ethically obtaining biopsies from patients early on in the course of AKI and CKD (61). One of the goals of 3D tissue cytometry in this project is to identify the spatial distribution of immune cell subtypes and their association with specific structures and neighborhoods during disease. In addition, linking these immune signatures to comprehensive molecular, pathology and clinical profiling will provide important advancements in understanding the pathogenesis of disease and improve disease classification based on refined cellular and molecular phenotypes (53, 55, 60, 61). Finally, we recently showed that using VTEA to profile the immune system in papillary biopsies of patients with kidney stones uncovered a unique signature of inflammation in brushite stone formers compared to calcium oxalate patients (62). Brushite papillae were characterized by increased neutrophil infiltration and neutrophil extracellular trap (NET) formation. In addition, neutrophil proteins were selectively enriched in the proteome of brushite stones, supporting a possible role of inflammation and NETosis in the pathogenesis of brushite stone formation (62). Such findings have implications on the approach and management of patients with nephrolithiasis.
**Tissue cytometry, big data and machine learning.**

Large scale 3D imaging and tissue cytometry surveys tens to hundred thousand cells. Out of these, the proportion and distribution of immune cells vary depending on the renal area but still likely be in the range of thousands (55). Multiplex labeling of a kidney tissue will increase the label-based features associated with a single cell and will exponentially increase the derived features based on label and spatial information. In fact, with seven markers, the resulting feature space just based on primary labels and spatial features already exceeds 50 variables per cell. As derived variables are added, we become faced with interrogating and visualizing hyperdimensional data for thousands of cells, a true “Big Data” problem. Although directed approaches and various sub-gating strategies can still be applied for specific variables, such methods would only allow a peripheral exploration of the data, and likely not capitalize on exploring the complex spatial relationships between various cells. Furthermore, an unbiased interrogation may not be possible using these traditional approaches. Therefore, to enhance the ability to analyze and visualize hyperdimensional data, various clustering and classification strategies using machine learning tools are being implemented in the VTEA analysis pipeline. These tools can allow the unbiased classification of cells based on multiple features (these new classes become themselves new derived features), visualization of hyperdimensional data using dimensionality reduction, and mapping cells back to the tissue based on these new derived features. Based on cell distributions within the tissue, cell neighborhoods can also be defined and quantitatively interrogated (36, 49) (Figure 4). These approaches are particularly useful for assessing immune cell involvement in pathology, including the
role of signaling in immune cell infiltration and the impact of immune cells on tissue injury or repair.

**In situ cell classification based on 3D nuclear staining using VTEA and deep learning**

Classifying cells type and subtypes based on imaging data typically requires validated markers. For example, subtypes of immune cells are characterized by the expression of unique cell surface markers (26, 63), and more recently also on defining transcriptomic profile (which still requires additional validation by RNA or protein detection) (29). Despite advances in multiplexing, a challenge remains that only a finite number of cell-associated markers can be obtained from a single experiment. In addition, each time a new marker is discovered or needed, new experiments on additional tissue sections are required. For sparse tissue such as a kidney biopsy or unique experimental conditions, this could lead to tissue exhaustion. Furthermore, because multiplexing is performed at the experimental level, re-classification of cells using prospectively discovered markers is not feasible except with new experiments, which will lead to missed opportunities for many already imaged kidney specimens.

Recently, we investigated whether 3D nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) (64), a nuclear stain commonly used in most fluorescence imaging methods contains enough information for reliable classification of human kidney cells *in situ* using a supervised learning framework (65). DAPI is commonly used in multiple platforms of research and can be easily performed as part of large-scale 3D imaging. In
addition, immune cells are frequently described based on their nuclear morphology. We first developed a machine learning approach using 3D cytometry with VTEA to generate a large training dataset comprising of 230,000 image volumes of nuclei (65), organized into cell types based on concurrent staining with validated markers (Figure 5). The development of such ground-truth library was only feasible because of the semi-automated workflow of VTEA, which mitigated costly efforts of manual annotation and allowed us to develop this library within few weeks. We then devised a CNN (Convolutional Neural Network) based deep learning approach to classify cells based on their 3D nuclear staining (Figure 5). Such an approach was successful in classifying 8 different cells types (various epithelial, immune and endothelial classes) with high accuracy (up to 84% balanced accuracy with maximal training), and then mapped back for visualization in the tissue (65). In our work we have used a standard CNN architecture for classification, but the accuracy of our proposed classification approach can likely be improved by developing custom-made deep learning methodologies. Furthermore, including concurrent staining with additional markers could potentially improve and expand such classification. Nevertheless, the promising results of our framework show that, imaging data from tissue stained with DAPI has a potential to become a rich source of data that will suffice for comprehensive cell classification. Furthermore, we envision that this methodology will have useful application for classifying immune cell subtypes. Combining cell classification with the VTEA cytometry interface will allow mapping of the identified cells and facilitate their biological interpretation in the context of tissue microenvironment. This unique approach enables the development of a ground truth library and the continued re-evaluation of existing
data, thereby lessening the need for more tissue. As new cell markers emerge, new training datasets can be generated from an abundant source of tissue. This learning can then be imputed on existing data sets from sparse tissue without the need for additional experimentation (Figure 6).

Future outlook, challenges and conclusions

In this review, we highlighted the emerging role of tissue cytometry in profiling immune cells within the kidney. New imaging and analytical tools, such as VTEA, have transformed imaging of kidney tissue into multidimensional “omics” data with rich information at the cell level. Such an approach will not only complement other innovative cell-based molecular technologies but will also help anchor the findings in situ and enhance the biological interpretation.

There are outstanding challenges for tissue cytometry, particularly in the areas of image processing, analysis and the computation and visualization of big data. For example, in the setting of disease, segmentation approaches need to be improved and refined to ensure accurate and robust data. Analytically, we need a thorough assessment of neighborhood approaches to better understand their application to the study of tissue microenvironments. Researchers also need the tools to explore and analyze the big data generated by national and international efforts such as the KPMP consortium through distributed cloud-based tissue cytometry platforms.

The output from tissue cytometry is expected to further increase, especially with advances in tissue clearing methods that allow mesoscale imaging (35, 49, 66).
Fortunately, advances in computational analysis and implementation of machine learning tools will enhance our ability to classify cells within the tissue and detect early changes in disease. For example, using the deep learning approach discussed above, it may become possible to detect early signatures of stress induced by disease and visualize the affected cells within the kidney using VTEA. Such advances will enhance the role of imaging and cytometry tools for discovery in nephrology.
Disclosures

S. Winfree reports Consultancy Agreements: QCDx LLC; Honoraria: University of Chicago, Marine Biological Laboratories, Course Instructor. T. El-Achkar reports Scientific Advisor or Membership: Frontiers renal pharmacology; Other Interests/Relationships: American Physiological Society, American College of Physicians, American Urological Association. The remaining author has nothing to disclose.

Funding

This work was supported by funding from the Veteran Affairs (Merit Award to T.M.E.), the National Institute of Diabetes and Digestive and Kidney Diseases (1R01DK111651 to T.M.E), and P30DK079312- Indiana University O'Brien Center for Advanced Renal Microscopic Analysis.

Acknowledgements

The authors would like to thank Drs. Katherine J. Kelly and Daria Barwinska for assistance in experiments and the Indiana Center for Biological Microscopy for imaging support in the Division of Nephrology and Hypertension at the Indiana University School of Medicine.

Author contributions

S Winfree: Conceptualization; Data curation; Formal analysis; Software; Writing - review and editing

M Al Hasan: Conceptualization; Writing - review and editing

T El-Achkar: Conceptualization; Data curation; Funding acquisition; Methodology; Writing - original draft; Writing - review and editing
References

1. Hato T, El-Achkar TM, Dagher PC: Sisters in arms: myeloid and tubular epithelial cells shape renal innate immunity. *Am J Physiol Renal Physiol, 304*: F1243-1251, 2013 10.1152/ajprenal.00101.2013

2. El-Achkar TM, Dagher PC: Tubular cross talk in acute kidney injury: a story of sense and sensibility. *Am J Physiol Renal Physiol, 308*: F1317-1323, 2015 10.1152/ajprenal.00030.2015

3. Kaissling B, Le Hir M: The renal cortical interstitium: morphological and functional aspects. *Histochem Cell Biol, 130*: 247-262, 2008 10.1007/s00418-008-0452-5

4. Soos TJ, Sims TN, Barisoni L, Lin K, Littman DR, Dustin ML, Nelson PJ: CX3CR1+ interstitial dendritic cells form a contiguous network throughout the entire kidney. *Kidney Int, 70*: 591-596, 2006 10.1038/sj.ki.5001567

5. Stewart BJ, Ferdinand JR, Young MD, Mitchell TJ, Loudon KW, Riding AM, Richoz N, Frazer GL, Staniforth JUL, Vieira Braga FA, Botting RA, Popescu DM, Vento-Tormo R, Stephenson E, Cagan A, Farndon SJ, Polanski K, Efremova M, Green K, Del Castillo Velasco-Herrera M, Guzzo C, Collord G, Mamanova L, Aho T, Armitage JN, Riddick ACP, Mushtaq I, Farrell S, Rampling D, Nicholson J, Filby A, Burge J, Lisgo S, Lindsay S, Bajenoff M, Warren AY, Stewart GD, Sebire N, Coleman N, Haniffa M, Teichmann SA, Behjati S, Clatworthy MR: Spatiotemporal immune zonation of the human kidney. *Science, 365*: 1461-1466, 2019 10.1126/science.aat5031

6. Teteris SA, Engel DR, Kurts C: Homeostatic and pathogenic role of renal dendritic cells. *Kidney Int, 80*: 139-145, 2011 10.1038/ki.2011.129

7. Chessa F, Mathow D, Wang S, Hielscher T, Atzberger A, Porubsky S, Gretz N, Burgdorf S, Grone HJ, Popovic ZV: The renal microenvironment modifies dendritic cell phenotype. *Kidney Int, 89*: 82-94, 2016 10.1038/ki.2015.292

8. Stamatiades EG, Tremblay ME, Bohm M, Crozet L, Bisht K, Kao D, Coelho C, Fan X, Yewdell WT, Davidson A, Heeger PS, Diebold S, Nimmerjahn F, Geissmann F: Immune Monitoring of Trans-endothelial Transport by Kidney-Resident Macrophages. *Cell, 166*: 991-1003, 2016 10.1016/j.cell.2016.06.058

9. Kolaczkowska E, Kubes P: Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol, 13*: 159-175, 2013 10.1038/nri3399

10. Nelson PJ, Rees AJ, Griffin MD, Hughes J, Kurts C, Duffield J: The renal mononuclear phagocytic system. *J Am Soc Nephrol, 23*: 194-203, 2012 10.1681/ASN.2011070680

11. Berry MR, Mathews RJ, Ferdinand JR, Jing C, Loudon KW, Wlodek E, Dennison TW, Kuper C, Neuhofer W, Clatworthy MR: Renal Sodium Gradient Orchestrates a Dynamic Antibacterial Defense Zone. *Cell, 170*: 860-874 e819, 2017 10.1016/j.cell.2017.07.022

12. Lever JM, Hull TD, Boddu R, Pepin ME, Black LM, Adedoyin OO, Yang Z, Traylor AM, Jiang Y, Li Z, Peabody JE, Eckenrode HE, Crossman DK, Crowley MR, Bolisetty S, Zimmerman KA, Wende AR, Mrug M, Yoder BK, Agarwal A, George JF: Resident macrophages reprogram toward a developmental state after acute kidney injury. *JCI Insight, 4*, 2019 10.1172/jci.insight.125503

13. Li L, Okusa MD: Macrophages, dendritic cells, and kidney ischemia-reperfusion injury. *Semin Nephrol, 30*: 268-277, 2010 10.1016/j.semnephrol.2010.03.005

14. Dong X, Swaminathan S, Bachman LA, Croatt AJ, Nath KA, Griffin MD: Antigen presentation by dendritic cells in renal lymph nodes is linked to systemic and local injury to the kidney. *Kidney Int, 68*: 1096-1108, 2005 KID502 [pii] 10.1111/j.1523-1755.2005.00502.x
15. Salei N, Rambichler S, Salvermoser J, Papaioannou NE, Schuchert R, Pakalniskyte D, Li N, Marschner JA, Lichtnekert J, Stremmel C, Cernilogar FM, Salvermoser M, Walzog B, Straub T, Schotta G, Anders HJ, Schulz C, Schraml BU: The Kidney Contains Ontogenetically Distinct Dendritic Cell and Macrophage Subtypes throughout Development That Differ in Their Inflammatory Properties. *J Am Soc Nephrol*, 31: 257-278, 2020 10.1681/ASN.2019040419

16. Chung AC, Lan HY: Chemokines in renal injury. *J Am Soc Nephrol*, 22: 802-809, 2011 10.1681/ASN.2010050510

17. Schlondorff D, Nelson PJ, Luckow B, Banas B: Chemokines and renal disease. *Kidney Int*, 51: 610-621, 1997 10.1038/ki.1997.90

18. Anders HJ, Ryu M: Renal microenvironments and macrophage phenotypes determine progression or resolution of renal inflammation and fibrosis. *Kidney Int*, 80: 915-925, 2011 10.1038/ki.2011.217

19. Zhang MZ, Yao B, Yang S, Jiang L, Wang S, Fan X, Yin H, Wong K, Miyazawa T, Chen J, Chang I, Singh A, Harris RC: CSF-1 signaling mediates recovery from acute kidney injury. *J Clin Invest*, 122: 4519-4532, 2012 10.1172/JCI60363

20. Watanabe S, Alexander M, Misharin AV, Budinger GRS: The role of macrophages in the resolution of inflammation. *J Clin Invest*, 129: 2619-2628, 2019 10.1172/JCI124615

21. Doran AC, Yurdagul A, Tabas I: Efferocytosis in health and disease. *Nature Reviews Immunology*, 20: 254-267, 2020 10.1038/s41577-019-0240-6

22. Chen T, Cao Q, Wang Y, Harris DCH: M2 macrophages in kidney disease: biology, therapies, and perspectives. *Kidney Int*, 95: 760-773, 2019 10.1016/j.kint.2018.10.041

23. Lee S, Huen S, Nishio H, Nishio S, Lee HK, Choi BS, Ruhrberg C, Cantley LG: Distinct macrophage phenotypes contribute to kidney injury and repair. *J Am Soc Nephrol*, 22: 317-326, 2011 10.1681/ASN.2009060615

24. Humphreys BD: Kidney injury, stem cells and regeneration. *Curr Opin Nephrol Hypertens*, 23: 25-31, 2014 10.1097/MNH.0000437332.31418.e0

25. Zimmerman KA, Bentley MR, Lever JM, Agarwal A: Mononuclear phagocyte subpopulations in the mouse kidney. *Am J Physiol Renal Physiol*, 312: F640-F646, 2017 10.1152/ajprenal.00369.2016

26. George JF, Lever JM, Agarwal A: Mononuclear phagocyte subpopulations in the mouse kidney. *Am J Physiol Renal Physiol*, 312: F640-F646, 2017 10.1152/ajprenal.00369.2016

27. Kurts C, Panzer U, Anders H-J, Rees AJ: The immune system and kidney disease: basic concepts and clinical implications. *Nature Reviews Immunology*, 13: 738-753, 2013 10.1038/nri3523

28. Micanovic R, Chitteti BR, Dagher PC, Srour EF, Khan S, Hato T, Lyle A, Tong Y, Wu XR, El-Achkar TM: Tamm-Horsfall Protein Regulates Granulopoiesis and Systemic Neutrophil Homeostasis. *J Am Soc Nephrol*, 26: 2172-2182, 2015 10.1681/ASN.2014070664

29. Zimmerman KA, Bentley MR, Lever JM, Li Z, Crossman DK, Song CJ, Liu S, Crowley MR, George JF, Mrug M, Yoder BK: Single-Cell RNA Sequencing Identifies Candidate Renal Resident Macrophage Gene Expression Signatures across Species. *J Am Soc Nephrol*, 30: 767-781, 2019 10.1681/ASN.2018090931

30. Hochheiser K, Heuser C, Krause TA, Teteris S, Ilias A, Weisheit C, Hoss F, Tittel AP, Knolle PA, Panzer U, Engel DR, Tharaux PL, Kurts C: Exclusive CX3CR1 dependence of kidney DCs impacts glomerulonephritis progression. *J Clin Invest*, 123: 4242-4254, 2013 10.1172/JCI70143

31. Micanovic R, Khan S, Janosevic D, Lee ME, Hato T, Srour EF, Winfree S, Ghosh J, Tong Y, Rice SE, Dagher PC, Wu XR, El-Achkar TM: Tamm-Horsfall Protein Regulates
Mononuclear Phagocytes in the Kidney. *J Am Soc Nephrol*, 29: 841-856, 2018 10.1681/ASN.2017040409

32. Liu J, Kumar S, Dolzenko E, Alvarado GF, Guo J, Lu C, Chen Y, Li M, Dessing MC, Parvez RK, Cippa PE, Krautberger AM, Saribekyan G, Smith AD, McMahon AP: Molecular characterization of the transition from acute to chronic kidney injury following ischemia/reperfusion. *JCI Insight*, 2, 2017 10.1172/jci.insight.94716

33. Brahler S, Zinselmeyer BH, Raju S, Nitschke M, Suleiman H, Saunders BT, Johnson MW, Bohner AMC, Viehmann SF, Theisen DJ, Kretzer NM, Briseno CG, Zaitsev K, Ornatsky O, Chang Q, Carrero JA, Kopp JB, Artyomov MN, Kurts C, Murphy KM, Miner JH, Shaw AS: Opposing Roles of Dendritic Cell Subsets in Experimental GN. *J Am Soc Nephrol*, 29: 138-154, 2018 10.1681/ASN.2017030270

34. Hato T, Zollman A, Plotkin Z, El-Achkar TM, Maier BF, Pay SL, Dube S, Cabral P, Yoshimoto M, McClintick J, Dagher PC: Endotoxin Preconditioning Reprograms S1 Tubules and Macrophages to Protect the Kidney. *J Am Soc Nephrol*, 29: 104-117, 2018 10.1681/ASN.2017060624

35. Gerner MY, Kastenmuller W, Ifrim I, Kabat J, Germain RN: Histo-cytometry: a method for highly multiplex quantitative tissue imaging analysis applied to dendritic cell subset microanatomy in lymph nodes. *Immunity*, 37: 364-376, 2012 10.1016/j.immuni.2012.07.011

36. Goltsev Y, Samusik N, Kennedy-Darling J, Bhat E, Hale M, Vazquez G, Black S, Nolan GP: Deep Profiling of Mouse Splenic Architecture with CODEX Multiplexed Imaging. *Cell*, 174: 968-981 e915, 2018 10.1016/j.cell.2018.07.010

37. Liarski VM, Kaverina N, Chang A, Yanez D, Talasnik L, Carlesso G, Herbst R, Utset TO, Labno C, Peng Y, Jiang Y, Giger ML, Clark MR: Cell distance mapping identifies functional T follicular helper cells in inflamed human renal tissue. *Sci Transl Med*, 6: 230ra246, 2014 10.1126/scitranslmed.3008146

38. Moreau HD, Lemaitre F, Terriac E, Azar G, Piel M, Lennon-Dumenil AM, Bousso P: Dynamic in situ cytometry uncovers T cell receptor signaling during immunological synapses and kinapses in vivo. *Immunity*, 37: 351-363, 2012 10.1016/j.immuni.2012.05.014

39. de Chaumont F, Dallongeville S, Chenouard N, Herve N, Pop S, Provoost T, Meas-Yedid V, Pankajakshan P, Lecomte T, Le Montagner Y, Lagache T, Dufour A, Olivo-Marin JC: Icy: an open bioimage informatics platform for extended reproducible research. *Nat Methods*, 9: 690-696, 2012 10.1038/nmeth.2075

40. Dobosz M, Ntziachristos V, Scheuer W, Strobel S: Multispectral fluorescence ultramicroscopy: three-dimensional visualization and automatic quantification of tumor morphology, drug penetration, and antiangiogenic treatment response. *Neoplasia*, 16: 1-13, 2014 10.1593/neo.131848

41. Ecker RC, Rogojanu R, Streit M, Oesterreicher K, Steiner GE: An improved method for discrimination of cell populations in tissue sections using microscopy-based multicolor tissue cytometry. *Cytotherapy*, 6: 119-123, 2004 10.1016/cyto.a.2001.05.014

42. Ecker RC, Steiner GE: Microscopy-based multicolor tissue cytometry at the single-cell level. *Cytotherapy*, 6: 119-123, 2004 10.1016/cyto.a.20052

43. Haubold C, Schiegge M, Kreshuk A, Berg S, Koethe U, Hamprecht FA: Segmenting and Tracking Multiple Dividing Targets Using Ilastik. *Adv Anat Embryol Cell Biol*, 219: 199-229, 2016 10.1007/978-3-319-28549-8_8

44. Kankaanpaa P, Paavolainen L, Tiitta S, Karjalainen M, Paivarinne J, Nieminen J, Marjomaki V, Heino J, White DJ: BioImageXD: an open, general-purpose and high-throughput image-processing platform. *Nat Methods*, 9: 683-689, 2012 10.1038/nmeth.2047

45. Ollion J, Cochennec J, Loli F, Escude C, Boudier T: Analysis of nuclear organization with TANGO, software for high-throughput quantitative analysis of 3D fluorescence.
microscopy images. *Methods Mol Biol.*, 1228: 203-222, 2015 10.1007/978-1-4939-1680-1_16

46. Peng H, Bria A, Zhou Z, Iannello G, Long F: Extensible visualization and analysis for multidimensional images using Vaa3D. *Nat Protoc.*, 9: 193-208, 2014 10.1038/nprot.2014.011

47. Rizk A, Paul G, Incardona P, Bugarski M, Mansouri M, Niemann A, Ziegler U, Berger P, Sbalzarini IF: Segmentation and quantification of subcellular structures in fluorescence microscopy images using Squashsh. *Nat Protoc.*, 9: 586-596, 2014 10.1038/nprot.2014.037

48. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A: Fiji: an open-source platform for biological-image analysis. *Nat Methods.*, 9: 676-682, 2012 10.1038/nmeth.2019

49. Stoltzfus CR, Filipek J, Gern BH, Olin BE, Leal JM, Wu Y, Lyons-Cohen MR, Huang JY, Paz-Stoltzfus CL, Plumlee CR, Poschinger T, Urda hl KB, Perro M, Gerner MY: CytoMAP: A Spatial Analysis Toolbox Reveals Features of Myeloid Cell Organization in Lymphoid Tissues. *Cell Rep.*, 31: 107523, 2020 10.1016/j.celrep.2020.107523

50. Wan Y, Otsuna H, Chien CB, Hansen C: FluoRender: An Application of 2D Image Space Methods for 3D and 4D Confocal Microscopy Data Visualization in Neurobiology Research. *IEEE Pac Vis Symp.* 201-208, 2012 10.1109/pacificvis.2012.6183592

51. Winfree S, Dagher PC, Dunn KW, Ferkowicz M, Barwinska D, Kelly KJ, Sutton TA, El-Achkar TM: Quantitative Large-Scale Three-Dimensional Imaging of Human Kidney Biopsies: A Bridge to Precision Medicine in Kidney Disease. *Nephron.*, 140: 134-139, 2018 10.1159/000490006

52. Winfree S, Ferkowicz MJ, Dagher PC, Kelly KJ, Eadon MT, Sutton TA, Markel TA, Yoder MC, Dunn KW, El-Achkar TM: Large-scale 3-dimensional quantitative imaging of tissues: state-of-the-art and translational implications. *Transl Res.*, 189: 1-12, 2017 10.1016/j.trsl.2017.07.006

53. Winfree S, Khan S, Micanovic R, Eadon MT, Kelly KJ, Sutton TA, Phillips CL, Dunn KW, El-Achkar TM: Quantitative Three-Dimensional Tissue Cytometry to Study Kidney Tissue and Resident Immune Cells. *J Am Soc Nephrol.*, 28: 2108-2118, 2017 10.1681/ASN.2016091027

54. El-Achkar TM, Huang X, Plotkin Z, Sandoval RM, Rhodes GJ, Dagher PC: Sepsis induces changes in the expression and distribution of Toll-like receptor 4 in the rat kidney. *Am J Physiol Renal Physiol.*, 290: F1034-1043, 2006

55. Ferkowicz M, Winfree S, Sabo A, Kamocka MM, Khochare S, Barwinska D, Eadon MT, Cheng Y, Phillips CL, Sutton TA, Kelly KJ, Dagher PC, El-Achkar TM, Dunn KW: Large-scale, three-dimensional tissue cytometry of the human kidney: a complete and accessible pipeline *Lab Invest.*, 2020

56. El-Achkar TM, Eadon MT, Menon R, Lake BB, Sigdel TK, Alexandrov T, Parikh S, Zhang G, Dobid D, Dunn KW, Otto EA, Anderton CR, Carson JM, Luo J, Park C, Hamidi H, Zhou J, Hoover P, Schroeder A, Joanes M, Azeloglu EU, Sealfon R, Winfree S, Steck B, He Y, D'Agati VD, Lyengar R, Troyanskaya OG, Barisoni L, Gatt J, Zhang K, Laszik Z, Rovin B, Dagher PC, Sharma K, Sarwal M, Hodgin JB, Alpers CE, Kretzer M, Jain S, The Kidney Precision Medicine Project F: A Multimodal and Integrated Approach to Interrogate Human Kidney Biopsies with Rigor and Reproducibility: Guidelines from the Kidney Precision Medicine Project. *Physiol Genomics.*, 2020 10.1152/physiolgenomics.00104.2020

57. Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, Guertin DA, Chang JH, Lindquist RA, Moffat J, Golland P, Sabatini DM: CellProfiler: image analysis software
for identifying and quantifying cell phenotypes. *Genome Biol*, 7: R100, 2006 10.1186/gb-2006-7-10-r100

58. Legland D, Arganda-Carreras I, Andrey P: MorphoLibJ: integrated library and plugins for mathematical morphology with ImageJ. *Bioinformatics*, 32: 3532-3534, 2016 10.1093/bioinformatics/btw413

59. LaFavers KA, Macedo E, Garimella PS, Lima C, Khan S, Myslinski J, McClintick J, Witzmann FA, Winfree S, Phillips CL, Hato T, Dagher PC, Wu XR, El-Achkar TM, Micanovic R: Circulating uromodulin inhibits systemic oxidative stress by inactivating the TRPM2 channel. *Sci Transl Med*, 11, 2019 10.1126/scitranslmed.aaw3639

60. Hansen J, Sealfon R, Menon R, Eadon MT, Lake BB, Steck B, Dobi D, Parikh S, Sidgel TK, Alexandrov T, Schroeder A, Otto EA, Anderton CR, Barwinska D, Zheng G, Rose MP, Shapiro JP, Velickovic D, Pamreddy A, Winfree S, He Y, de Boer IH, Hodgin JB, Nair A, Sharma K, Sarwal M, Zhang K, Himmelfarb J, Laszik Z, Rovin B, Dagher PC, He JC, El-Achkar TM, Jain S, Troyanskaya OG, Kretzler M, Iyengar R, Azeloglu EU: Towards Building a Smart Kidney Atlas: Network-based integration of multimodal transcriptomic, proteomic, metabolomic and imaging data in the Kidney Precision Medicine Project. *bioRxiv*: 2020.2007.2023.216507, 2020 10.1101/2020.07.23.216507

61. de Boer IH, Alpers CE, El-Achkar TM, Azeloglu E, Balis UG, Barasch J, Barisoni L, Blank K, Bombaek AS, Brown K, Dagher PC, Dighe AL, Eadon MT, Gaut J, Hacohen N, He Y, Hodgin JB, Jain S, Kellum JA, Kirdlug K, Knoght R, Laszik Z, Lienczewski C, Mariani LH, Mccielland RL, Menez S, Moledina D, Mooney SD, O'Toole J, Palevsky P, Parikh CR, Poggio E, Rosas S, Rosengart MR, Sarwal M, Schaub JA, Sedor JR, Sharma K, Steck B, Toto R, Troyanskaya OG, Tuttle KR, Vazquez M, Waikar SS, Williams K, Wilson FP, Zhang K, Iyengar R, Kretzler M, Himmelfarb J: The Kidney Precision Medicine Project: rationale and design. *Kidney Int*, 2020

62. Makki MS, Winfree S, Lingeman JE, Witzmann FA, Worcester EM, Krambeck AE, Coe FL, Evan AP, Bledsoe S, Bergslund KJ, Khochare S, Barwinska D, Williams JC, Jr., El-Achkar TM: A Precision Medicine Approach Uncovers a Unique Signature of Neutrophils in Patients With Brushite Kidney Stones. *Kidney Int Rep*, 5: 663-677, 2020 10.1016/j.ekir.2020.02.1025

63. Bohner AMC, Viehmann SF, Kurts C: Unraveling the Complexity of the Renal Mononuclear Phagocyte System by Genetic Cell Lineage Tracing. *J Am Soc Nephrol*, 31: 233-235, 2020 10.1681/ASN.2019121295

64. Kapuscinski J: DAPI: a DNA-specific fluorescent probe. *Biotech Histochem*, 70: 220-233, 1995 10.3109/10520299509108199

65. Woloshuk A, Khochare S, Almulhim AF, McNutt AT, Dean D, Barwinska D, Ferkowicz MJ, Eadon MT, Kelly KJ, Dunn KW, Hasan MA, El-Achkar TM, Winfree S: In Situ Classification of Cell Types in Human Kidney Tissue Using 3D Nuclear Staining. *Cytometry A*, 2020 10.1002/cyto.a.24274

66. Klingberg A, Hasenberg A, Ludwing-Portugall I, Medyukhina A, Mann L, Brenzel A, Engel DR, Figge MT, Kurts C, Gunzer M: Fully Automated Evaluation of Total Glomerular Number and Capillary Tuft Size in Nephritic Kidneys Using Lightsheet Microscopy. *J Am Soc Nephrol*, 28: 452-459, 2017 10.1681/ASN.2016020232
Figure legends

Figure 1. Overview of tissue cytometry with VTEA. A. Two or three-dimensional multichannel images of fluorescently stained tissue, including DAPI and cell markers, are imaged by high resolution microscopy. B. These datasets are imported into VTEA and processed for noise, background correction, etc. and segmented into individual cells based on DAPI stained nuclei. C. Segmented nuclei are processed and the intensity of the pixels surrounding each nucleus are measured and summarized statistically. D. Per segmented nuclei, the summary statistics and other image features can be plotted, gated and quantitated. E. Gated cells are assessed and confirmed in tissue volume by dynamic mapping-back of selected cells to the imaged volume. F and G. Within the imaged volume, a region-of-interest can be added to determine where spatially localized cells are found in the analytic space. H. These groups of cells can be used to classify cells for use in additional unsupervised and neighborhood analyses.

Figure 2. Profiling immune cells in mouse kidneys following ischemic injury using 3D tissue cytometry. A. Whole kidney section stained for MHCII (magenta) and with DAPI (nuclei, gray) and phalloidin (F-actin, yellow), was imaged by confocal microscopy with tile-scanning after which image volumes were stitched together. A single optical slice is shown. Inset (a) at right is a maximum z-projection from the region indicated. Scale bars = 1 mm (A) and 100 microns (a). B. Cells were segmented and quantitated with VTEA yielding 124,548 cells. A gate was used to identify MHCII+ cells that were mapped back to the tissue (left and middle panel respectively) and presented in the same volume given in inset (a) from panel A (right panel). Scale bar = 1 mm.
Figure 3. Quantitation of immune cell infiltrate in reference nephrectomy tissue.
Fixed human cortical nephrectomy tissue was stained with antibodies against CD45, CD31, and Nestin and with DAPI. This tissue was imaged by confocal fluorescence microscopy and analyzed with VTEA. All images are maximum z-projections. A. Stitched confocal volumes with glomeruli (Nestin, magenta), a network of endothelial cells (CD31, yellow) and infiltrating immune cells (CD45, green) throughout the tissue. Scale bar = 100 microns. B. Immune cells are associated with and away from CD31+ cells-see inset (a). (Scale bar = 50 microns). C. Tissue cytometry quantitatively identifies immune cells (CD45+) near CD31+ cells (red gate) or far from CD31+ cells (cyan gate). D and E. Overlay of gated cells from panel C highlights the distributions of the two populations.

Figure 4. Incorporating spatial context with neighborhood analysis. A. Cells are first classified by using either a supervised gating strategy or an unsupervised clustering approach. B. These classified cells are grouped into non-exclusive overlapping neighborhoods by neighborhood building algorithms such as space dividing (left panel) or cell centric approaches such as cells within a given distance or the k-nearest neighbors of a reference cell (center and right panels respectively). C. The composition of these neighborhood can be interrogated based on classification and connectivity with network analyses.
Figure 5: Workflow for *in situ* classification of cells using 3D nuclear staining:

This approach combines tissue cytometry (VTEA) with deep leaning using a convolutional neural network. VTEA allows ground truth validation of cell types and subtypes based on specific markers and generation of ground truth library. Currently, such library encompasses 270,000 image volumes of nuclei for 8 different classes of cells. This ground truth library allows the training, validation and testing of a deep learning approach to classify cells based only on the 3D nuclear staining pattern. The classified cells can then be mapped back in the tissue with VTEA to visualize and interpret the biological context.

Figure 6. Multiplexed imaging of sparse tissue: challenges and solutions. A. a typical workflow for large scale imaging and analysis. As novel markers emerge, there is a need to re-stain or incorporate markers into a congested processing pipeline with new experiments. This process is limited by the number of markers used and will deplete the tissue as new markers become available. B. implementing VTEA with deep learning to classify cells based on 3D nuclear staining. The nuclear signature of new cell types can be validated in abundant sources of tissue and imputed on the sparse tissue. This allows the extraction of novel data from already imaged sparse tissue and provides a non-exhaustive source for discovery with benefits on tissue economy.
Figure 3
Figure 4

A. Classification

B. Neighborhood building

Space dividing

Distance

k-Nearest (k=5)

C. Analysis and visualization

Cell classification

| Neighborhood | N1 | N2 | N3 | N4 | N5 |
|--------------|----|----|----|----|----|
| S1           |    |    |    |    |    |
| S2           |    |    |    |    |    |
| S3           |    |    |    |    |    |
| CD45+LY6G+   |    |    |    |    |    |

Heatmaps

Graphs
Figure 5

1. Staining kidney tissue with specific cell markers
2. Tissue Cytometry identifies specific cell types
3. Ground Truth Validation in situ
4. Ground Truth Library Generation using VTEA
5. Training, validation and testing of classification using deep learning
6. VTEA used for visualizing cell classes and link back to the biology in situ
Figure 6

**Standard Approach**

A

- Tissue used once
- More tissue needed
- New marker

B

- Tissue used once

**VTEA + Deep learning**

- Ground-truth library
- Non-exhaustive
- Imputed classification

Sparse tissue