Title: Visualizing Metabolic Diversity, Precision, and Patterning

Running Head: Visualizing Metabolic Diversity and Precision

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Abstract

The metabolic profiles of distinct cell classes are difficult to capture in tissue contexts. We have developed and validated a technology that interrogates four fundamental attributes of individual cells of heterocellular tissues: (1) cellular diversity and (2) patterning within tissues, (3) cell-specific metabolite levels and (4) variability. Computational molecular phenotyping (CMP) combines ultra-thin tissue section arrays, small molecule immunodetection, high-resolution imaging, and pattern recognition to extract high-dimensionality metabolic signatures from all cells, no matter how rare, in tissues of complex organisms.

Anti-hapten immunglobulin G & Y (IgG & IgY) antibody libraries were used to quantitatively probe ultrathin section arrays for small molecules. Molecular signals were imaged, tiled into large mosaics, and registered into multichannel data sets. Pattern recognition was used to build classification maps, differentiating statistical classes of cells, and allowing N-dimensional visualization of identified cell signatures.

CMP visualization permits three primary findings. (1) Metabolic diversity is high in heterocellular systems. Distinct metabolic profiles are partitioned into discrete cell classes (metabolons) in heterocellular tissues. Both cell-intrinsic and cell-interactive mechanisms (e.g. coupling) generate diversity. (2) Metabolic precision is high within cell classes. (3) Metabolons have topologies, ranging from strongly gridded cell mixtures (e.g. neuroglial and muscle systems), discrete serial modules (pulmonary and renal assemblies), large-scale coupled gradient systems (liver) and nominally un-patterned cell mixtures (endocrine and immune tissues).

Unlike unicellular models, heterocellular systems concurrently display diversity and precision. The metabolic variability of unicellular organisms commonly studied in metabolomics (yeast, E. coli) is
likely not representative of single-class precision in complex metazoan tissues, where metabolite levels can be clamped by interacting loops of transport, synthesis and sequestration. Further, supracellular architectures such as vascularity and cell patterning clearly shape metabolon topologies, which in turn likely reflect spatially patterned small molecule chemistries. CMP offers the prospect of metabolic profiling in virtually all organisms and expands the spectrum of biomarker candidates and target systems for complex diseases such as cancer and neurodegenerations.

Introduction
Towards cellular metabolomics
A metabolon is an array of cells serving connected metabolic functions in a tissue compartment [1]. A metabolome is a quantitative small molecule profile that cells in a metabolon use to negotiate physiologic operations such as osmoregulation, pH regulation, reactive oxygen species buffering, polymer assembly, signaling, energy conversion and storage, group transfer and proliferation. These are re-entrant definitions, since a meaningful description of a tissue metabolome requires prior knowledge of metabolon composition (cell classes, numbers, distributions, adjacencies, coupling), while characterizing metabolon operations requires deep knowledge of metabolome compartmentalization. No dominant technologies for acquiring this information from heterocellular systems have emerged.

Much of our current knowledge of metabolic networks largely focuses on unicellular models and modularity within rather than across cells [1,2,3,4], and does not predict how metabolon architectures and cell patterning evolved, nor does it illuminate selective advantages of heterocellular compartmentalization. Mendes [5] has articulated many of the technical challenges of metabolomics, including the need for advanced profiling and analytic tools. One pressing need is high spatial resolution metabolic mapping [6]. Most metabolomics analyses target unicellular systems.
[2,7], biofluids [8] or homogenized tissues [9] and cannot readily explore spatially compartmentalized metabolite distributions, cellular diversity, cell patterning and number, or intercellular dependencies. Further, metabolomics studies often focus on end-stage or unique pathway products rather than common core pathway intermediate signatures. Though formal, large-scale networks of core metabolites seem stereotyped across the biota [2,10], fine-scale core metabolite synthesis, transport, conversion and storage processes are clearly compartmentalized across different cell classes in metazoan tissues. Cell, metabolon and tissue boundaries matter and we seek technologies that can visualize these boundaries in the context of small molecule patterning. The key is high spatial resolution imaging.

Anti-hapten technologies for metabolomics

Interrogating metabolic profiles is challenging issue. How should small molecules be trapped, derivatized, detected and/or mapped into cellular space? There are several technologies for tracking small molecules in at cellular resolution: microdissection [11], imaging mass spectrometry (IMS) [6], riboswitch engineering [12], and FTIR microscopy [13]. However, the barriers to adoption of these methods are several, including lack of general applicability (riboswitches, microdissection), unspecified biomarker identities (IMS, FTIR), limited molecular scope (riboswitches, FTIR), limited resolution (IMS, microdissection) and immense expense (IMS, FTIR). However, a stable profiling technology has largely flown ‘under the radar’ of cell and molecular biology. Metabolite signals can be quantitatively mapped with hapten-specific IgG reagents that detect free or conjugated small molecules with high fidelity and signal strengths proportional to free intracellular levels [14,15,16,17]. When combined with multispectral imaging and pattern recognition, such computational molecular phenotyping (CMP) enables precise visualization multiple metabolites in every cell [14,18,19]. CMP provides the ability to concurrently discover and phenotype cell classes,
track cell state, and map disease or challenge sequelae with single-cell resolution in any tissue or organism.

### Table 1  IgGs and IgYs Targeting Small Molecules

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Key: IgG, rabbit immunoglobulin G; IgY, chicken immunoglobulin Y; SICOM, signature immunoligics, Inc., Salt Lake City, UT; MarcLab, The Marc Laboratory, John A. Moran Eye Center, University of Utah School of Medicine, Salt Lake City, UT.

Landsteiner’s early demonstrations of regio- and stereospecific binding of small molecules by IgGs [20] long remained unexploited in cellular mapping until it was revived and enhanced by Ottersen [15,21], and discrimination of even enantiomers (e.g. D- and L-aspartate) is now routine [14,22,23]. Though hapten immunochemistry has largely been used to probe immune mechanisms
or generate immunoassays, targeting free aromatic [24,25] and aliphatic amines [14,15,17,26] to probe cellular expression patterns has become standard in neurobiology.

Many small molecules can be targeted by hapten detection methods. Even exotic polymeric targets such as C60 fullerenes, targets as nonpolar as cholesterol, or as large as vitamin B12 have been ‘haptenized’ [27,28,29]. Extremely high affinities can be tuned for steroid targets by site-directed mutagenesis [30]. Most biologically relevant small molecules are potential haptens and we have assembled a large library of probes targeting small molecules (Table 1).

Tracking core metabolites
Using anti-hapten IgGs and IgYs, we have mapped core metabolite signatures in over 30 tissue types, in over 70 organisms, in all vertebrate classes as well as selected plants, invertebrates and fungal cells. These data show that virtually all heterocellular tissues display core metabolite diversity across and high precision within cell classes and/or states in the case of unicellular organisms. This paper focusses on mammalian tissues. Most tissues were probed for a core metabolite subset: L-alanine (A), L-aspartate (D), L-glutamate (E), glycine (G), glutathione (J), L-glutamine (Q), L-arginine (R), taurine (τ) and 4-aminobutyric acid (γ, GABA). This vector basis set (abbreviated A.D.E.G.J.Q.R.τ.γ, analogous to a true vector basis, a linearly independent spanning set) interrogates common cellular activities: glutathione probes reactive oxygen species control; taurine probes osmoregulation; arginine is modulated with protein synthesis; DEQ (aspartate-glutamate-glutamine) profiles core glutamate metabolism; CH3 group transfer and possible glycine synthesis is probed by alanine; GABA marks inhibitory signaling or shunt metabolism. A small but growing library of antibodies targeting pivotal proteins (e.g. glutamine synthetase) can also be embedded in these sets. This basis set readily segments cells into metabolic classes. It includes core metabolites that are the most vertebrate common group transfer and homeostatic molecules. Glutamate and
glutamine in particular are maintained at tissue concentrations 10-100 fold higher than necessary for protein synthesis and form critical metabolic nodes in the tissues of vertebrates. Glutamate is involved in over a dozen synthetic pathways (Fig. 1), making it one of the central currencies of core metabolism. Immunodetection of this basis set of primary amines is highly effective. Amines can be trapped by dialdehyde fixation with ≈ 85% efficiency [14] and high precision. Shared signatures of homologous cell classes across species shows that trapping is reproducible [14,18,19,31,32,33]. Extensive exploration with this basis set has already proven effective in retina [12], brain [34,35], lung [36]. Furthermore, the basis set can easily be changed and signatures augmented with new probes at nearly any time.

Small molecules were probed on ultrathin serial section surfaces, detected with gold-coupled IgGs, and visualized with silver intensification [14,37], providing a detection range of ≈ 50 μM – 10 mM [18]. High-resolution optical images were tiled into mosaics and registered to create data-

Figure 1. The multiple metabolic pathways emanating from glutamate and glutamine nodes. Black circles denote molecules or pools for which CMP probes exist. White circles indicate probes in development. Probes for which both D and L-isomer specific IgGs exist are marked with asterisks. Key: 1 glutamate, 2 pyruvate, 3 alanine, 4 oxalacetate, 5 asparate, 6 2-oxoglutarate, 7 glutamine, 8 UTP, 9 CTP, 10 xanthosine-5’-P, 11 guanosine-5’-P, 12 α-D-5-P-ribosyl-PP (PR-PP), 13 PR amine, 14 PR glycaminide (GAR), 15 N-formyl GAR, 16 glutamyl-P, 17 glutamate semialdehyde, 18 ornithine, 19 glucosamine, 20 fructose-6-P, 21 lactate, 22 4 aminobutyrate, 23 succinate semialdehyde, 24 histidine, B glutamine-fructose transaminase, GS glutamine synthetase, PAG P-activated glutaminase, P phosphate, Urea Cycle, Ur uridine pools, Cy cytidine pools, Xa xanthine pool, Gu guanosine pool, PPS pentose P shunt, Pu purine ribonucleotides, PA polyamine degradation, pro protein synthesis, PD protein degradation, GAD glutamate decarboxylase, U, arginine synthesis, Z glutathione synthesis, M multiple glutamine transaminases.
sets where each spatial coordinate indexes an N-space chemical vector. Molecular diversity is readily viewed with monochrome or rgb-mapped imaging. For example, $\tau QE :: rgb$ maps taurine, glutamine and glutamate channels to red, green and blue display channels, respectively. Cell-specific molecular diversity and signal dispersions were extracted by clustering and visualized as theme maps that guide exploration of metabolic classes corresponding to natural cell classes.
RESULTS

A framework for gauging cellular metabolic diversity

Every cell class has a different tissue role reflected in its diverse but stable small molecule signature. In most cases the differences are large. Figure 2a displays a \( \tau QE :: rgb \) mapped image of the cat retina. The \( \tau QE \) signature is especially valuable as \( \tau \) signals reveals cells that must manage strong osmotic challenges; \( Q \) signals reveals supportive cells that expressly synthesize glutamine on behalf of dependent cells; \( E \) signals represent core neurotransmission and precursor pools. This small signature alone is able to segment the retina into at least seven metabolically distinct superfamilies (Fig. 2B) that are known to transfer carbon skeletons in a heterocellular network, including neuroglial glutamate \( \leftrightarrow \) glutamine exchange cycles, and separate organic ion osmoregulation networks that may be partial cycles. Though taurine is clearly an obligate osmolyte for retina \[38,39,40\], there is no evidence that it is synthesized there. Thus part of the signature diversity arises from the compartmentalization of cells into four metabolically and osmotically distinct compartments (sensory retina, neural retina, neural vascular bed, choroidal vascular bed). However this does not explain all diversity as several metabolic “styles” clearly exist within the neural retina alone, with yellow \( \tau Q^+ \) Müller cells, magenta \( \tau E^+ \) bipolar cells, cyan \( QE^+ \) ganglion cells, and a spectrum of amacrine cell signals (there are over 30 amacrine cell classes in the mammalian retina). The important lesson from this signature profile is that diversity is obvious, even in a small metabolite space. A less obvious lesson is that any arbitrary signature is insufficient to display the full extent of the biologic diversity. Upon expanding the signature to the seven member \( A \cdot D \cdot E \cdot G \cdot Q \cdot \tau \cdot \gamma \) set, we see that the simplistic cyan \( QE \) signal of retinal ganglion cells vastly underestimates the metabolic diversity of the retinal ganglion cell layer alone. Figure 3 displays rgb, monochrome and theme maps of the ganglion cell layer in adult rabbit retina, revealing a kaleidoscope of coexisting metabolomic styles. Traditional tissue/biofluid profiling yields only the weighted average of cellular concentrations, with the bulk variance over time or individuals arising
from occult, weighted diversity of metabolic styles. The $\tau$QE map (Fig. 3A) segments the image into the three broad groups that dominate the ganglion cell layer: (i) yellow-orange glial Müller cells, (ii) cyan ganglion cells that dominate the cell bodies in the layer, and (iii) purple-black local circuit neurons (amacrine cells). The GyD map (Fig. 3B) unmask complex GABA and glycine content.
patterns due to heterocellular coupling in the ganglion cell superclass and a large-cell bias for maintaining high aspartate pools. For example, class 3 and 9 have nearly indistinguishable $\tau_{QE}$, but vastly different $G\gamma D$ signatures. Conversely, classes 1 and 2 have rather similar $G\gamma D$ but dissimilar $\tau_{QE}$ signatures. The class 9 group is actually a collection of at least four different kinds of GCs that can’t be split based on this signature alone, but can be with the addition of activity markers [18]. CMP is an effective tool for discovery when a broad basis set is used.

It is difficult to sort cells in such images by visual inspection, since a seven channel basis set produces 35 unique rgb images, and humans cannot see variance. The simplest and most fundamental solution is pattern recognition via unsupervised classification by K-means or isodata clustering, producing a theme map that indexes all the unique statistical classes of elements in a scene [14,18,41]. Other segmentation methods such as Principal Components Analysis are also valuable (and sometimes faster with large datasets), but largely converge on the same solutions. Clustering segments this basis set of ganglion cell layer signals into ten unique groups including the Müller cells, two amacrine cell classes, and seven ganglion cell superclasses. After classification, each class may be interrogated with more advanced histogramming tools. One simple way to visualize such high-dimensional data is via stacked 2N plots [18] where several data pairs are viewed in 2D space (Fig. 3E), similar to cell-sorting imagery. Different cells can be readily distinguished by their distinctive “paw prints” in molecular space. Class signatures reveal both extreme and subtle core metabolite signature diversities (see Fig. S1) beyond the scope of this manuscript, but the central message we wish to convey is that there is no single metabolome for all cells and tissues.

Metabolic diversity and precision across tissues.
Metabolic diversity is not unique to neural systems and CMP readily unmasks different cell classes in many tissues. An ultrathin coronal CMP series through the eye of a mouse visualized with $\tau_{QE}$
Fig. 4. Diversity and precision of glutamine levels. (A) τQE map of a mouse eye showing concentric signatures: (a) sclera, (b) choroid, (c) RPE, (d) rod outer segments (ROS), (e) rod inner segments (RIS), (f) outer nuclear layer, (g) distal inner nuclear layer, (h) amacrine cell layer, (i) inner plexiform layer, (j) ganglion cell layer, (k) optic fiber layer, (ON) optic nerve. The arc indicates the direction of a stepped glutamine scan path around the eye. (B) Enlargement of box B, panel A showing paths through the RPE (1,3), vascular lumens (2), ROS (4,6), RIS (5). (C) Enlargement of box C in panel A showing inferior oblique myocytes with high (1) and medium (2) glutamine levels. (D) Enlargement of box D, panel A showing medial rectus myocytes with low (3) glutamine levels. (E) Glutamine pixel values (PV) around the eye: grey, unfiltered signal; black, 8 μm boxcar average. (F) Normalized histograms of PN vs PV from class 1, 2, and 3 myocytes; (G) Histograms of absolute log10 PN vs PV. Scales: A 0.1 mm; B 0.2 mm; C,D 0.01 mm.
mapping (Fig. 4A-D) concurrently samples core signatures from vascular, muscular, adipose, connective, transport, and neuroglial tissues, producing over a dozen separable signatures in addition to the neural retina. Many metabolic styles emerge, including E-rich vascular endothelia, τQ-rich transport retinal pigmented epithelium (RPE), τE-rich rod photoreceptor ellipsoids specialized for metabolic conversions, various functional groupings of muscle fibers with unique τ-biased profiles, low-level Q signals throughout the sclera, and a great variety of QE mixtures in retinal neurons. Neighboring compartments do not predict each other’s signatures, though each manifests local precision exemplified by the orderly gradation of Q signals across cells (Fig 4E). A continuous trace of glutamine signals through over 250 RPE and 600 photoreceptor cells shows that RPE cells always maintain higher glutamine levels than photoreceptors. Subtle variations in mean RPE glutamine levels are small compared to the total ocular range and are mostly technical (see methods and [12]). Class diversity extends to nominally homogenous tissues such as muscle (Fig. 4C,D,F,G). Even the inferior oblique and medial rectus muscles of the eye have significantly different signatures, and this includes specialized small cells. These myocytes cluster into classes 1, 2 and 3 based on increasing glutamine content. Centrally positioned inferior oblique class 3 myocytes possess higher glutamine levels than any other ocular myocytes (Fig. 4C, F, G), yet comprise <10% of total muscle glutamine and far less than 0.01% of ocular glutamine. Their uniquely tuned metabolisms could never be found by tissue measures. Why is this important? Such myocytes may be progenitor cells and their unique metabolisms may be part of the mechanisms underlying their transformation into abnormal proliferating cell groups in ocular rhabdomyosarcomas.

Topology and Patterning.
CMP also reveals that every metabolon has a functional topology. Neural tissues such as retina are built upon gridded metabolons: mixtures of diverse and independently patterned (17,18) cell
Fig. 5. Diverse metabolic signatures and cell patterning in muscle fibers (A) GQE map of mouse intercostal muscle showing a mosaic of signatures. Superclass 3 cells show a distinctive high Q signal and a set of five cells is bounded by Voronoi domains. Panel A is 1.087 mm wide. (B) A theme map of seven metabolic classes shown as a mirror-image with the corresponding myocytes indicated in A. (C) Absolute histograms of GQE signatures for all myocytes of classes 1.1, 2.1, 3.1 and 3.2. Labeling conventions as in Fig 3. (D) A scatterplot of superclasses 1 (G+), 2 (E+) and 3 (Q+) with circle areas corresponding to myocyte cross-sectional area, glutamine concentration on the log_{10} ordinate, and the glycine/glutamate ratio on the log_{10} abscissa.
classes where cell numbers and spacing are tightly regulated [42]. Robust patterning also exists in skeletal muscle. In mouse intercostal muscle visualized by GEQ mapping (Fig. 5A), clustering clearly segments the tissue into three superclasses: superclass 1 with G+ large fibers, superclass 2 with E+ intermediate fibers, and superclass 3 with Q+ small cells. Each superclass can be split into two or three classes of a metabolic manifold (Fig. 5B). Superclass G+ (1) is glycine-rich, has large
Fig. 7. Gradient metabolite patterns in mouse liver. (A) Concurrent GS.Q.E :: rgb mapping in liver revealing GS hot-spots (red-orange) in perivenous (V) domains centered in clouds of glutamine-rich (green) parenchymal hepatocytes in a sea of glutamate-rich/glutamine-poor parenchymal and periportal (P) hepatocytes. Max-min contrast stretched. (B) The same data set viewed as raw, unstretched channels. The white trace is the signal path used to profile hepatocyte contents. (C) The GS signal (green) isolated on a magenta glutamine (Q) background. (D) The isolated glutamine signal (green) viewed on a magenta DAPI background. (E) The isolated GS signal (green) viewed on a magenta DAPI background. (F) The isolated glutamate signal (green) viewed on a magenta DAPI background. Image, 0.82 mm wide and 2.0 mm high.
merically dominant (>60% of the fibers and 80% of the mass) and contains the class manifold 1.1, 1.2, and 1.3. Superclass E+ (2) is glutamate-rich, medium sized, represents about 25% of the cells and less than 15% of the mass and contains classes 2.1 and 2.2. Superclass Q+ (3), as in ocular muscle, is composed of very small glutamine-rich cells, again representing a little over 10% of the muscle cells, less than 10% of the mass, and contains classes 3.1 and 3.2. The signature histograms for classes 1.1, 2.1, 3.1 and 3.2 (Fig. 5C) demonstrate the very low dispersion of metabolite concentration around its mean. Figure 4D is a 4-dimensional plot that displays glutamine concentration, the glycine-glutamate ratio and fiber diameter. The three superclasses are clearly segregated. Importantly, the spatial distribution of class Q+ fibers is highly patterned, whether assessed by Voronoi areas (Fig. 5A) or conformity ratios (the ratio of the mean and standard deviations of the nearest-neighbor distances). In this sample, the conformity ratio is 2.7, which approaches $p \approx 0.001$ using the significance tables of Cook [43].

Pulmonary (Fig. 6A) and renal (Fig. 6B) tissues represent serial modular systems where cell classes with different metabolic styles are spatially segregated. JπE mapping in mouse lung (Fig. 6A) dem-

Fig. 8. Gradient metabolite profiles in mouse liver. (A) Pixel value profiles for GS (red) and Q (green) viewed along the 1.3 mm path in Figure 7B. (B) Pixel value profiles for GS (red) and E (blue) viewed along the 1.3 mm path in panel B. (C) Molar E/Q ratios along the profiled path. V, perivenous zones; P periportal zones. Pixel values were converted to concentrations using the silver equation (see Methods). Ratios were set to zero over the portal lumen.
Fig. 9. Mixtures of metabolite profiles in spleen. (A) DE\(\tau\) mapping in mouse spleen with highly varied signature mixtures in functional reticular zones (B), follicular zones (C) and follicle → marginal zone transitions (D). Panel A is 2.114 mm wide. (B) Reticular zone and perivenous sinuses with a central vein (arrow) and clusters of highly DE+ lymphocytes (double arrows). (C) White pulp follicle with densely packed DE+ lymphocytes. (D) An arc of D+ lymphocytes extending from the edge of the follicle into the marginal zone. The very thin \(\tau\)-rich serosal layer is indicted by an arrow. (E,F,G) Aspartate images corresponding to panels B, C and D. Panels B-G are 0.25 mm wide.
onstrates that high glutathione signals are expressed by cells closely clustered in bronchiolar modules dominated by ciliated cells and non-ciliated Clara cells [36], whereas high glutathione levels are restricted to sparse subsets in alveolar modules. Further, vascular elements possess their own distinctive high τE signals. Mouse renal cortex displays over six distinct metabolic styles in the serial modules forming the vascular pole, glomerulus, urinary pole, proximal and distal convoluted tubule systems, and collecting ducts (Fig. 6B).

In liver, a mainstay of whole-tissue metabolomics, the fundamental tissue module is a collection of cells spanning venous and portal drainages [44]. Perivenous hepatocytes preferentially express glutamine synthetase (GS) [45] while glutamate-producing glutaminase expression is biased towards periportal hepatocytes [46]. Parenchymal hepatocytes bridging these zones are coupled by Cx26 and Cx32 homo- and heterotypic connexons [47] permeable to small molecules. This forms bidirectional transcellular glutamine and glutamate gradients uniquely visualized by QJE mapping (Fig. 7) in sections fortuitously aligned with the mouse liver unit module. Whether this gradient represents truly normal in vivo patterning or an emergent patterning resulting from transient ischemia in fixation cannot be determined yet, but such gradient patterns are not seen in other systems with extensive coupling such as retina. In any event, the ability to visualize small molecule gradients arising from the spatial separation of sources and sinks opens new opportunities to explore the roles of coupling and local transcriptional regulation in the assembly of organ systems. By tracing a path across liver modules, it is obvious that both the glutamine surplus (Fig. 8A) and glutamate deficit (Fig. 8B) zones extend beyond the GS-source cells. Further, the glutamate/glutamine ratio across the same gradient spans a decade (Fig. 8C). This suggests that liver is an ideal model for exploring the mechanisms of spatial metabolite control, especially in terms of pathways for connexon modulation of space constants for metabolite diffusion through coupled parenchymal hepatocytes.
Endocrine pancreas and immune tissues such as lymph nodes and spleen (Fig. 9) represent complex mixtures where no obvious gridded, serial or gradient modules are readily detected. Immune tissues represent some of the most daunting mixtures of metabolic styles we have yet encountered and stereological CMP reconstructions are clearly called for. It is clear that the core signatures of immune cells in spleen are diverse. In both splenic and circulating lymphocytes, aspartate signals are high and taurine signals low. Conversely, taurine signals are high in macrophages, platelets and polymorphonuclear cells (not shown) and glutathione signals are enriched in the mouse spleen marginal zone, and may be associated with marginal metallophilic macrophages (ref). Clustering
Fig. 11. 2N plot signatures for over 1100 individual cells grouped into 9 ocular cell classes (rods, A HCs, B HCs, MCs, RPE, ScCs, ChCs, RBCs, ECs and VCs - vertical cells of the choroid) or 5 superclasses (BCs, on cBCs, GCs, gly ACs, γ ACs). Each coordinate spans PV 0-255 and crosses at PV 127 (nominally 0.34 mM), spanning a -1 to 1 log_{10} concentration scale (0.1-10 mM). Each signature is made up of three bivariate clouds centered on the class mean ± 2SD, coded as xy pairs: YE (grey); GJ (cyan); QT (gold). In column 3, single normal MC (blue arrows) is compared with the MC class and a single pathologic J+ MC.
suggests that over a dozen superclasses of cells exist in the spleen.

**Metabolic precision**

Given that there are so many different classes of cells, we wondered how robust they were. Thus we sought to explore the precisions of metabolic tuning in different cell types class. CMP allows concurrent measures of all cells, no matter how rare, so a single sample can be mined extensively. Figure 9 is a fragment of a large ocular region in which over 1100 cells were analyzed as a EGjQTγY set. Sixteen different cell classes or superclasses representing neurons, glia, epithelium, endothelium, blood elements and fibroblasts were analyzed by CMP and signatures generated for each class (Fig. 10,11). Each class/superclass signature was visualized as a 2N-plot devised by Marc and Jones (2002). This scheme compresses 6 or more channels into 2-space, displaying the molecular “pawprint” of each class as 3 bivariate clouds of 2SD diameter centered on the bivariate mean. The remarkable and intriguing outcome is that every class/superclass is absolutely distinct and separable from every other. Each is so precisely tuned that no other distinct cell class can be confused with it and each encompasses only a tiny part of tissue space. In fact, much of the vari-
ability in some classes is clearly technical: alignment errors in small cells, intracellular protein texturing, slice-to-slice organelle misplacements, subtle lighting inhomogeneities, camera noise, silver enhancement noise. The true metabolic precision within a class is certainly higher than indicated here.

Different metabolites and cells show different absolute variations Fig. 11. For example, the 95% confidence limits for 100 rod photoreceptors spans 2-2.3 mM for glutamate and 230-290 µM for glutamine. The confidence limit for glutathione in 100 RPE cells is 1.8-2.3 mM, almost twice that of photoreceptors. The much larger variations for bipolar cells, amacrine cells, and ganglion cells arise from the fact that each is a superclass containing 10-30 distinct classes. Even so, no other cell class would be confused with them. There are some obvious patterns, such as the unique association of high GABA and glycine levels with inhibitory neurons. Conversely, one can’t easily assign cell function to a glutamate level. Retinal neurons such as GCs and structural fibroblasts such as sclerocytes have remarkably similar glutamate contents (both in the 2-5 mM range) but have clearly different functions. And retinal Müller cells contain almost the same low amount of glutam-
mate as red blood cells (Müller cells, 340-380 µM, RBCs 230-250 µM) but for very different reasons. Glia have massive glutamate transport and oxidative metabolism, but even greater glutamate conversion via glutamine synthetase. RBCs lack significant glutamate formation and use most of that for glutathione synthesis (≈ 1 mM). It appears that there are no distinct forbidden metabolite mixtures. The significances of the precision concept include (i) validating biofluid measures as accurate cellular measures (e.g. RBCs) and (ii) the ability to detect variations from normality in a given cell class. Importantly, one can always find at least one signal for every cell class pairing where the histograms have such little overlap that \( P < 0.0001 \) (Fig. 13). This is a crude but effective way of demonstrating the power of N-space separability statistics [14,18].

This demonstration of precision suggests model of metabolome partitioning and regulation (Fig. 14). The concentration of a molecule in a cell reflects the nature of its availability to different cellu-
lar processes. Small molecule encounter rates at lateral translation speeds are vastly different across concentration regimes. Below 0.3 mM, small molecules are widely spaced (root mean square spacing of 1180 nm at 1 µM) and will have low encounter rates for sparse targets like enzyme active or regulatory sites. Thus they are likely engaged in sparse operations such as protein synthesis and modulatory cofactor roles, where essential elements such as transfer RNA can be pre-charged and stored. We term this the *sparse regime*, as events can be critically limited by small molecule availability. Between 0.3 and 1 mM, (rms spacing of 12 nm at 1 mM), metabolite levels far exceed those required for protein synthesis, but match enzyme affinities and meet demand for the high-availability group transfers that define core metabolism. We term this the *reactive regime*. Above 1 mM, (rms spacing of 5.5 nm at 10 mM), small molecules are used as high-flux signals (neurotransmitters), colligative processes such as osmoregulation (taurine, myoinositol) or semi-colligative properties such as buffering (taurine, glutathione). We term this the *flux regime*. Though the regime borders are arbitrary and fuzzy, the absolute availability of small molecules clearly alters the nature and scale of their functions. At the same time, the precision of the metabolome differs in each regime. If we display the dispersion of a molecule’s measured content within a class of cells as the histogram full-width at half height (FWHH, data from Fig. 2), we discover that metabolite variation is a saturating function of content. In the sparse regime, dispersion is proportional to the mean class signal, which implies substantial noise: regulation is limited by sparseness. In the flux and reactive regimes, the saturation relation implies that control effects limit the impact of noise sources. Again, it is important to stress that the dispersion we measure is itself significantly increased by technical noise, that metabolic precision is likely far higher within a class than we can yet measure.
DISCUSSION

CMP offers new strategies to explore the phylogenetics of metabolons, discover novel cell classes (27) and track rare classes (28) invisible to biofluids analyses. The remarkably low metabolic variances of individual cell classes suggests that sources of metabolic noise are few; that each cell tightly regulates metabolite formation, conversion and transport. In addition to metabolic network control (9), low variance may also represent precise control of protein expression. For example, all ocular myocytes likely possess the exactly the same metabolic network, and small but stable differences in synthetic or transport protein levels would readily account for their metabolic precision and stable differences from other cells. Expression noise varies greatly across proteins in unicellular organisms, and some metabolic proteins in particular show low variation [48]. Metazoans may display stronger precision in protein expression within a cell class than unicellular populations. But, even so, there is much to learn from unicellular models that may impact heterocellular design. For example, retinal Müller cells are extremely large and complex, with multiple functional compartments. How Müller cells manage sparse regime metabolite supply in the face of wide intracellular variation in ribosome patterning may be better understood using novel concepts such as translation on demand and just-in-time translation [49]. Though intercellular coupling in complex tissues is a source of metabolic diversity across cells, it clearly does not impair signal precision within classes, implying that connexin expression is also precise. Uncoupling can produce metabolic noise, however, and may be an early pathologic signature in many tissues. For example, retinal detachment stress evokes uncoupling and anomalously high metabolic diversity in Müller glia signatures (20), suggesting that tissue-based measures of increased metabolite variance could be a sensitive disease signature.

Cellular metabolomics is a new player in a rapidly advancing field with powerful, large-scale molecular profiling tools [50], burgeoning correlative databases [51], and high impact in diagnos-
tics and identifying lead compounds for drug discovery [52,53]. In this context, the narrow molecular scope of CMP is clearly problematic but not insurmountable. New advances in imaging mass spectrometry (IMS) now permit determinations of molecular diversity at near-cellular resolution (6). Conversely, CMP is unmatched in spatial precision and many unique probes remain to be generated. Further, CMP uniquely allows visualization of common core metabolite patterning and subcellular compartments (13) and is compatible with ultrastructural mapping (21). IMS, biofluids screening and CMP are clearly complementary, and data from one could profitably steer the others, offering new strategies to define richer models of tissue function.

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MATERIALS AND METHODS

Animals
Mice were obtained from The Jackson Laboratories and rabbits from Western Oregon Rabbitry. All methods for anesthesia and euthanasia conform to institutional animal care and use authorizations at the authors’ respective institutions and the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and have been detailed in previous publications [18,36].

Tissue harvesting and sample processing
All tissues and were fixed by immersion, maintained (overnight to indefinitely) in buffered 2.5% glutaraldehyde, 1% formaldehyde, and processed for epoxy resin embedding as previously described. Single or stacked samples were serially sectioned at 40-200 nm onto 12-spot Teflon®-coated slides (Cel-Line; Erie Scientific Inc), fully sodium ethoxide etched, probed with IgGs targeting various molecules, and visualized with silver-intensified 1.4 nm gold granules conjugated to goat anti-rabbit or goat anti-mouse IgG (Nanoprobes, Yaphank, NY). Immunoreactivity is a pure surface phenomenon independent of section thickness as illustrated in Fig. S2. Small molecule IgGs (anti-L-alanine [IgG A], anti-L-aspartate [IgG D], anti-L-glutamate [IgG E], anti-glycine [IgG G], anti-glutathione [IgG J], anti-L-glutamine [IgG Q], anti-taurine [IgG τ], anti-GABA IgG γ) were provided by Signature Immunologics Inc (Salt Lake City, UT). Anti-glutamine synthetase was obtained from BD Transduction Laboratories (Cat # 610517).

Probe selectivity and calibration
Anti-hapten IgGs were calibrated for selectivity on known standards by (i) immunoblots; (ii) competition curves based on bis-amino conjugates of glutaraldehyde (GA) as displacers (e.g. E-GA-E, γ-GA-γ, etc.) summarized for the major core probes in Table S2; and (iii) by cross-correlation of signals on tissues. The competition curves show that, under tissue conditions, there should be no
detectable cross contamination. This is supported by cross-correlation of tissue signals. For example, in Fig. 1, 2 and S3, E signals are completely independent of γ signals and vice versa over nearly two decades. The bidirectional E⋅Q gradients in liver (Fig. 7) show that the two IgGs cannot detect each other's targets in the biological range. Similar pairwise non-correlations can generated from biological samples for all the IgGs used in this study, though this cannot be exploited in every instance. For example, there is a slight positive correlation of A and G values in retina because alanine is likely the immediate precursor of glycine for many cell classes. Even so, IgG G is $10^7$-fold selective for G over A and IgG A is $10^3$-fold selective for A over G (Table S2). Quantitative scalings were calibrated from immunoblot detection ranges and validated for key molecules on artificial standards. These were generated as mixtures of 15% ovalbumin, 100 mM lysine and varying amounts of free amines on 0.1 M phosphate buffer pH 7.4, trapped with 250 mM glutaraldehyde until gelled and laminated as multi-sample specimens that were processed, sectioned and probed on slides just as tissues were probed. A representative glutamate standard spanning 0.3 - 5 mM is shown in Fig. S4. It is possible that our concentration estimates are low due to tissue losses but, as we have shown previously, our estimated integrated concentrations correspond closely to whole tissue concentrations estimated from HPLC [14].

**Imaging**

All data were captured as 8-bit 1388 pixel x 1036 line frames under voltage regulated tungsten halogen flux with a variation of 1.2 ± 0.6%/min (mean ± sd). Images were captured with a Peltier-cooled QImaging Fast 1394 QICAM (QImaging, Burnaby, BC, Canada) and autotiled with Surveyor 5.5.5.22 (Objective Imaging, Cambridge, UK) and a Scan 100x100 stage (Märzhäuser Wetzlar GMBH & Co, Germany). Images were analyzed without filtering or scaling. Final tiling and multi-modal registration was performed with IR-tweak, a multi-platform registration application written by P. Kolshvoy, (University of Utah School of Computing). IR-tweak is an interactive application for
pairwise image registration based on a Thin Plate Spline transform and exploits OpenGL texture memory and fragment shader capabilities of graphics cards standard on modern computers. More information about IR-tweak and other related microscopy image processing applications can be found at http://www.sci.utah.edu/download/ncrtoolset.

Analysis and Display

Individual cell classes or superclasses were extracted from monochrome images by K-means clustering using pixel-based (PCI Remote Sensing, Inc., Richmond Hill, Ontario, Canada) (S1, S2) and object-based algorithms (CMPView V1.0, University of Utah, © J.R. Anderson) built in MATLAB 7.2 (The MathWorks, Natick Massachusetts). Cluster classes were visualized as theme maps. Univariate (S1) and bivariate 2N plot (S2) pixel probability density histograms of metabolite signals in each cell class were extracted with CellKit V2.4OSX (University of Utah, © R. Marc) built in the IDL programming language (ITT Visual Information Solutions, Boulder, CO). Each histogram represents all of the possible 8-bit pixel values associated with a class and is proportional to log$_{10}$ estimated concentration (S1, S2, S4). Each univariate histogram was smoothed with a 5 bin Lee filter. Bivariate histograms were unfiltered. Adobe® PhotoShop® CS3 Beta Public Release was used for final image generation. Display images were generated by linear contrast stretch of each channel using minimum pixel values of 30 and maximum pixel values of 120-220.

Acknowledgments

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Supporting Online Material

Table S1: $\log_{10}$ relative ligand required to block tissue binding

Legend. Top row, IgG type; left column, competitor. Each IgG was used at the optimal dilution for quantitative detection (previously determined from saturation assays) on a standard section of retinal tissue used to calibrate all IgGs. Dilutions: IgG A (1:10000), IgG D (1:2000), IgG E (1:32000), IgG G (1:4000), IgG J (1:4000), IgG Q (1:2000), IgG $\tau$ (1:16000), IgG $\gamma$ (1:32000). Competitors were created by adding 2 moles of free amine / mole of purified glutaraldehyde in a pH 7.4 0.1M phosphate buffer medium, resulting in a nominal bis-amine ligand mimicking 2 molecules of tissue antigen/molecule competitor. Competitors were applied over a 12 $\log_{10}$ unit range with IgGs to standard samples, visualized with CMP, and the concentration required for 100% blockade if IgG binding to known cellular targets determined for each IgG-competitor pair. The absolute blocking concentrations for the cognate pairs (e.g. IgG A vs A-GA-A) were: A 1 nM, D 1 pM, E 100 nM, G 1 pM, J 10 pM, Q 1 nM, $\tau$ 10 nM, $\gamma$ 10 nM. The cognate values were set to 0 and the differentials with other pairs tabulated. For example, the competitor J-A-J displaces IgG J 10$^9$-fold more effectively than any other ligand. For tissue level detection, this constitutes virtually absolute selectivity: a 5 mM tissue level of glutathione generates a high level of IgG J binding and very strong signal in cells. To generate any contamination of that signal, glutamate or any other competitor would have to be present at physiologically unreachable levels. Similar arguments hold for the entire set.

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Figure S1. (A) Normalized univariate histograms for 10 cell classes across 7 metabolite channels (left column, classes 1-5; right column, classes 6-10). Ordinate: normalized pixel number. Abscissa: 8 bit pixel value (PV) spanning 0.1-10 mM on a $\log_{10}$ scale. Curved lines trace modal signals for selected classes across all metabolites. The yellow trace compares GABAergic amacrine cells (class 4) with a strongly coupled ganglion cell (class 3, cyan trace). The red trace compares weakly coupled ganglion cells (class 7) with Muller cells (class 10, blue trace). Crossing traces reveal major diversities in core profiles. (B) Bivariate histograms 2N-plots for all 10 classes. Each plot represents four signal pairs (AG black, DQ cyan, $E\tau$ magenta, $E\gamma$ gold), each pair mapped as 8-bit PV on xy coordinates (e.g. A=x, G=y). The color shading represents the density of pixels with that bivariate value and the vector lines connect the nominal 1 mM origin with the cloud peak. Each plot spans estimated concentration on a -1 to 1 $\log_{10}$ scale (0.1-10 mM). The top two rows display the signatures of ganglion cells (classes 1,2,3,5,6,7,9) and the combined vector lines for all classes are summarized in plot V. The minimum convex hull spanning each bundle of vectors forms a gan-
glion cell “pawprint” radically different from amacrine cell (classes 4,8) and glial (class 10) signatures. (C) Simplified flux and content schematic reflecting (i) cell-intrinsic conversions (e.g. Q → E), cell-intrinsic transport (e.g. Q export and import), (ii) cell-extrinsic microenvironments (extracellular E, Q, γ) and (iii) cell-interactive transfers (coupling conductances -^_^^-; extracellular short arrows denote synaptic release ). Müller glia (MC) are the prime source of all retinal Q, exporting it to all neurons. They are also the prime sink for E released by photoreceptors and bipolar cells (BC) and γ released by amacrine cells (AC) via high-affinity transporters. All neurons synthesize E largely from Q and amacrine cells convert E to γ.

Fig. S2. Signal strength is independent of section thickness. A series of six sections spanning 680 nm was initiated with a 40 nm optical section and each successive section was increased in thickness by 40 nm (40, 80, 120, 160, 200, 240 nm). Three sections from that series are shown. All were probed concurrently with the IgG E and visualized in the intensity mode. The images are derived directly from raw data without filtering or contrast adjustment and represent sections spanning a 5-fold difference in thickness. The PV density profile (red trace ranging from a minimum of 116 to 255) was taken along the black arrow for all sections. Each section is statistically indistinguishable from its neighbor.

Fig. S3. Cross-correlation proves the independence of IgG signals. Glutamate (E) and GABA (γ) signals from all image loci in Fig. 1 were plotted in a bivariate space and visualized as an equalized density histogram plot with dark signals representing high frequency signal pairs and lighter orange to white representing less frequent. The fact that values spread across the entire concentra-
tion domain means that there is no predictive correlation between the two. In fact, the high density ridges run parallel to the axes. This is direct evidence that no significant cross reactivity can exist between the two IgGs. Similar demonstrations exist for most pairs.

Fig. S4. Silver visualization intensity (inverse density) is log-linear with concentration. A. A 250 nm thick section of a laminated concentration strip containing 4.0, 2.0, 1.0, 0.5, 0.25 and 0.125 mM glutamate linked to a protein matrix by glutaraldehyde, probed with IgG E, detected by silver intensification, and visualized as a raw 8-bit density image (left strip) and a max-min linearly contrast stretched image (right strip). B. The mean intensity (inverse density) pixel value (PV) of each concentration on the strip is plotted against log concentration (black data points). As the signal nears 255, density begins to saturate and the calibration deviates from log-linearity. However, atomic silver emits visible light upon excitation (S5), and concurrent emission/density curves allow partial correction for nearing density saturation (yellow data points). The data are superimposed on a first-order standard saturation function: \( P = \frac{(C \cdot P_{\text{max}})}{(C + \sigma)} \) where \( P \) is pixel value, \( P_{\text{max}} = 255 \), \( C \) is concentration, and the half-saturation constant = 0.3 mM.
SOM references


