Advances in Toponomics Drug Discovery: Imaging Cycler Microscopy Correctly Predicts a Therapy Method of Amyotrophic Lateral Sclerosis

Walter Schubert*

Abstract
An imaging cycler microscope (ICM) is a fully automated (epi)fluorescence microscope which overcomes the spectral resolution limit resulting in parameter- and dimension-unlimited fluorescence imaging. This enables the spatial resolution of large molecular systems with their emergent topological properties (toponome) in morphologically intact cells and tissues displaying thousands of multi protein assemblies at a time. The resulting combinatorial geometry of these systems has been shown to be key for in vivo/in-situ detection of lead proteins controlling protein network topology and (dys-)function: If lead proteins are blocked or downregulated the corresponding disease protein network disassembles. Here, correct therapeutic predictions are exemplified for ALS. ICM drug target studies have discovered an 18-dimensional cell surface molecular system in ALS-PBMC with a lead drug target protein, whose therapeutic downregulation is now reported to show statistically significant effect with stop of disease progression in one third of the ALS patients. Together, this clinical and the earlier experimental validations of the ICM approach indicate that ICM readily discovers in vivo robustness nodes of disease with lead proteins controlling them. Breaking in vivo robustness nodes using drugs against their lead proteins is likely to overcome current high drug attrition rates.

Key terms
ALS; functional superresolution; imaging cycler microscopy; toponome; MELC; high content analysis; drug discovery; topology

Definitions
Toponomics is a discipline in systems biology, molecular cell biology, and histology (1). It concerns the study of the toponome of organisms. The toponome is the spatial network code of proteins and other biomolecules in morphologically intact cells and tissues, including fluid tissues such as blood. The term toponome is derived from the ancient Greek nouns topos (topos), place, and nomos (nomos), law, and thus a descriptive term addressing the fact that the spatial network of biomolecules in cells follows topological rules enabling coordinated actions (1,2). The information contained in a toponome relies on intact tissues.

Microscopic Method for the Study of Molecular Systems
Why Resolve Molecular Systems Spatially (Toponomics)?
Traditionally, tissue analysis is performed by using microscopic methods. It is the daily routine in histology and histopathology labs. Without histopathology clini-
ing disease process?

therapeutic modulation could interfere with the correspond-

pathology and directly derive molecular components whose

aligning "molecular systems histology" with traditional histo-

visualize such networks directly in a tissue section thereby

do they look like? Further, which substructures hold these net-

mal histopathological structures and disease phenotypes. How

which molecular tissue networks generate normal and abnor-

coordinates and collective functional properties of their

tems can be considered as having tissue-specific spatial

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methods enabling us to interrogate tissues in a way that we can

the myriad of hitherto unknown structural and func-

tional systems units driving diseases inside tissues. These sys-

can be considered as having tissue-specific spatial
coordinates and collective functional properties of their

molecular components. How assess such systems and discover

which molecular tissue networks generate normal and abnor-

mal histopathological structures and disease phenotypes. How
do they look like? Further, which substructures hold these net-

works together at specific tissue site? Finally, is it possible to

visualize such networks directly in a tissue section thereby

aligning "molecular systems histology" with traditional histo-

pathology and directly derive molecular components whose

therapeutic modulation could interfere with the correspond-

ing disease process?

Technological Basis and Proof of Concept

I deal with this problem since 1987 and since that time

have collected many tissue types in order to analyse and

compare large molecular systems in tissue sections and in

peripheral mononuclear blood cells (PMBC) (4). Finally, this

led to the discovery of disease specific rules and the general

principles of molecular systems organisation in vivo

(1,2,4,6). For this purpose an automated device had to be

developed (7), today termed imaging cycler microscope

(ICM) (8). An ICM is a fully automated (epi)fluorescence

microscope which overcomes the spectral resolution limit

resulting in parameter- and dimension-unlimited fluorescence imaging (Figs. 1 and 2). The principle and robotic
device was described in 1997 (7) and has been further
developed with a team and with co-workers within the

human toponome project (6,8–11). The ICM runs robotically

controlled repetitive incubation-imaging-bleaching cycles on

a specifically fixed biological sample with dye-conjugated

probe libraries (antibodies, lectins, peptides or any specific

affinity reagents) on stage of an epifluorescence microscope
to co-localize large numbers of different biomolecules in situ. (for technology real time insight see www.huto.topo-

nomos.com). This results in the transmission of a randomly

large number of distinct biological informations by re-using

the same fluorescence channel, e.g., for FITC imaging, if

FITC is conjugated to each probe, where each probe has its

unique specificity. Thereby noise-reduced quasi-multi chan-

nel fluorescence images with reproducible physical, geo-

metrical, and biophysical stabilities are generated. The resulting

power of combinatorial molecular discrimination (PCMD)

per data point is given by, e.g., 65,536 (4), where 65,536 is the

number of grey value levels (output of a 16-bit CCD camera)

and k is the number of co-mapped biomolecules and/or

many subdomains per biomolecule(s). High PCMD has been

shown for k = 100, (4,6,10) with 256 100 and in principle can

be expanded for much higher numbers of k. In contrast to

traditional multi-channel-few parameter fluorescence micro-

scopy (Fig. 1b) high PCMDs in an ICM lead to high

functional and spatial resolution (Fig. 1a). Systematic ICM

analysis of biological systems reveals the supramolecular seg-

regation law that describes the principle of order of large,

hierarchically organized biomolecular networks in situ (topo-

onome) (5). The ICM is the core technology for the system-

atic mapping of the complete protein network code in
tissues (human toponome project) (11). The original ICM

method (7) includes any modification of the bleaching step.

Corresponding modifications have been reported for anti-

body retrieval (12) and chemical dye-quenching (13)
debated recently (8,14). The toponome imaging systems

(TIS) and multi-epitope-ligand cartographs (MELC) repres-

ent different stages of the ICM technological development. I

have found that the sunlight-similar straylight conditions

used in the ICM set up for imaging and bleaching is the

method of choice not altering the tissue integrity "on stage"
over long periods of time (review in (4)). Moreover, repeti-
tive cycling is important for diluting out unspecific binders

(15) to yield specific and highly resolved multi molecular

biomolecular assemblies (Figs. 1 and 2). Many ICM based

studies revealed that molecular networks of membrane asso-
ciated biomolecules are hierarchically organised with lead

proteins controlling protein network topology and function:

when the lead protein is blocked or inhibited, the corre-
sponding functional protein network disassembles leading to

loss of (dys)function [4–6]. ICM based lead-protein detec-
tion and prediction as molecules hierarchically controlling

pathogenic molecular networks was confirmed experiment-
tially in mouse models for ALS (16), chronic neuropathic
pain and in cultured tumour cells [6,(17)]. Imaging Cycler Microscopy received the American ISAC best article award in 2008 for the three symbol code of organized proteomes [18], by which large three-dimensional protein networks can be imaged per cell (Fig. 1e) or 2D molecular networks can be detected for diagnostic purposes (Fig. 3). In extension to the latter threshold based code, a non threshold based real time molecular profiling, based on mathematical theorems [19,(20)], enables the investigator to directly visualise large multimolecular assemblies, e.g., associated with the basal lamina (Fig. 1a), or detect protein profiles that sharply distinguish between normal and diseased tissue at the same time [4] (Fig. 2). Cyclical imaging has been applied to address a variety of biological problems [21–54], and

Figure 1. Functional super resolution of large molecular networks. (a-d) Dermoeipithelial junction in human tissue. Imaging cycler microscopy based discovery of molecular networks in situ (4). (a) Direct realtime protein profiling in 100-dimensional ICM data set using an algorithm based on the similarity mapping approach (19,20,22). Each data point has a PCMD of 256^100. Note: sharp images at the junctional area discriminating between Lamina Fibroreticularis (LF), Lamina Densa (LD, green profile in d), Lamina Lucida (LL) and the basal ceratinocyte layer (BC, red profile in d), as known from transmission electron microscopy (c). (b) Same area as in (a), displaying traditional triple fluorescence imaging. (e) 3-dimensional ICM imaging of distinct 32-component multi protein complexes on the cell surface of a blood T-lymphocyte (8). Multi protein complexes are composed of differential combination of 32 proteins/glycotopes listed in (f). (g) Examples are marked with asterisks (number 1 to 3) and detailed as CMPs with proteins present (1) or absent (0) together characterised as individual CMPs. Bars: 10 μm (a, b), 50 nm (c), 1 μm (e). A similar figure is featured in http://www.toposnomos.com/huto/tis.html. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Figure 2. Discovery of disease specific 100-dimensional protein profiles simultaneously and in real time in morphology intact tissue sections at a PCMD of 256100 per pixel, exemplified in human skin. (a) List of 100 co-mapped biomolecules and selected protein profiles (0, 1, 3, 32 – 35) specific for diseased (d, f) and normal skin (e, g). (b, c) Diseased (b) and normal skin (c) are highlighted by pseudo colouring as histological stain for morphological orientation. Note that, by moving the cursor over the pixels, the software directly recognises in real-time which protein profiles are specific for the diseased (d, f) or normal skin (e, g). For example, pixel protein profiles (PPP) with numbers 0 and 1 are specific for the normal skin (e), and PPP 3, as well as PPPs 32 – 35 (d, f, respectively) are specific for the diseased skin. Note: For many similar applications at real time see webpage of the human toponome (HUTO) project (www.huto.toposnomos.com). (h) Power law (Zipf’s law) substantiates highly organised protein systems, as seen in (d-g). If 49 molecules are co-mapped Zipf’s law applies (blue line), but does not apply, if <15 molecules are co-mapped (red and green lines). This is revealed by plotting the log-log-relationship of thousands of distinct protein assemblies in toponome data sets (5,6). Bar: 100 μm (b–g). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
mathematics/informatics approaches to ICM data have been published (19,20,52,55–57). Several editorials (58,59) and a research highlight (60) have featured toponome research.

ICM–BASED APPROACH TO DISEASE ROBUSTNESS

Amyotrophic Lateral Sclerosis: the Predictive ALS Toponome

ALS is a fatal disease of the motor neuron system leading to steadily progressive muscle weakness. Today it is commonly accepted that the clinical phenotype of the disease is the consequence of multiple pathogenic processes, involving motor neurons and surrounding non-neuronal cells, as well as myocytes (61). Over 50 clinical trials have been conducted in ALS over a period of 25 years. With exception of Riluzole trial, all have failed (62). Looking back to the 1990es, a multisystem cause of ALS was not discussed. I have started to address cell surface associated multi-protein clusters in peripheral blood mononuclear cells (PBMC) in ALS between 1990 and 1995 using the technology later termed Imaging Cycler Microscopy (ICM) (see above). I was prompted to analyze the ALS cellular immune system by ICM, because I saw a young patient aged 18 diagnosed as ALS with supranuclear palsy showing a severely altered lymphocyte homeostasis in PBMC with almost complete absence of CD8 T cells. This patient died 6 months after diagnosis of ALS. I interpreted this finding likely to reflect a substantial intrinsic alteration of the cellular immune system rather than merely a reaction to a neurodegeneration, and I took a closer look at the immune cell differentiation. I initiated a first quantitative four-parameter screening study (63). We found highly unusual if not abnormal immune cell subsets in a number of ALS patients expressing CD16+ CD8+ and CD57+ or CD16+ CD8+ CD57– cell surface clusters only in sporadic ALS with supranuclear palsy and in sporadic ALS with predominant involvement of the upper motor neuron. As an association of CD8 and CD16/CD57 in PBMC cell surface membranes is usually not seen in normal blood, the data suggested to look more precisely into the higher order cell surface differentiation, i.e., co-organization of proteins of the cellular immune system in ALS-PBMC using the ICM principle for co-mapping a large number of different cell surface proteins. By overcoming the fluorescence spectral resolution limit using ICM (described above) it was possible to resolve an 18-dimensional molecular system in ALS peripheral blood mononuclear cells in 1999 as described in several international patents (64–66). This molecular system, referred to as the ALS toponome or ALS motif, is hierarchically organized and revealed CD16 (Fc[RIII]) as lead protein and therapeutic target (64–66). The ALS motif contains CD16 and CD45RA as lead proteins denoted (1 = lead protein, present in all CMPs) (d, motif M1) (64–66). Figure 3a shows presence of this ALS motif only in ALS PBMC, but not in normal control PBMC (Fig. 3b). This functional prediction of CD16 lead protein as therapeutic drug target cluster is now clinically substantiated: well tolerated downregulation of CD16 in a phase I clinical trial (67) is associated with beneficial effects in 27% of ALS patients with stop of disease progression in a phase II clinical trial over a 6 month period, which reached statistical significance when compared to concurrent and historical placebo controls (62). This proof of concept substantiates the predictive power of large molecular systems detected by ICM in toponome-based drug discovery, and shows that parameter-unlimited ICM imaging in intact human tissues is a reliable condition for correct drug target predictions. Imaging the corresponding molecular network as a combinatorial molecular phenotype (CMP) motif (ALS CMP motif) (Fig. 3d) reveals its presence only in ALS PBMC (Fig. 3a). Further, as therapeutic downregulation of the lead-protein CD16 (and dysfunctions associated with this downregulation), as predicted [64–66], results in a beneficial clinical effect [67], the

Figure 3. ALS specific motif as predictive toponome biomarker visualized simultaneously in ALS PBMC (a) as directly compared with healthy control (b). The motif was revealed by fully automated ICM based co-mapping of 18 cell surface proteins (c) on isolated PBMC. The motif as a whole is composed of 200 distinct CMPs. The visual field of (a) shows cells, each of which displays one ALS-specific CMP (different colours). The motif contains CD16 and CD45RA as lead proteins denoted (1 = lead protein, present in all CMPs) (d), while other proteins are either not-associated (0 = anticolocated), or variably associated with the lead proteins (** = wild cards) (d, motif M1) (64–66). Courtesy of HUTO Project. Bar: 100 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
shown ALS motif (Fig. 3a) is a robustness network of ALS, whose stability can be broken by interfering with its lead protein. The wide angle of view is a necessary condition to visualize most of the CMPs of this motif (robustness network) simultaneously. CD16 is present in all cells expressing a given CMP of the ALS motif suggesting that early detection of this pathological motif can be helpful or decisive for diagnosis and early disease management in cases of suspected disease.

Together, ICM correctly identified a molecular disease network of ALS and a lead protein controlling it (64–66). The method of blocking the lead protein to block its pathogenic function can be a direct (64) or an indirect one (65,66). The latter indirect method (65,66) was substantiated experimentally in a CD16 KO mouse (16) having implicated that therapeutically blocking the specific binders of CD16 (the IgG1 and IgG3 proteins) as published earlier (64–66) and thereby preventing them from binding to the corresponding CD16 binding sites is a valid method indirectly inactivating the pathogenic function of CD16 leadprotein and thus is a promising way for clinical therapeutic intervention in ALS. These two direct and indirect principles [(64–66), respectively] paved the way and stimulated the use of specific other molecules to downregulate functions of CD16 expressed in cellular systems in ALS, directly or indirectly, and many studies have confirmed CD16 as critical marker in ALS (68). In particular, NP001 was applied and reported to efficiently downregulate CD16 (67) as critical biomarker and control element of therapeutic success. Other molecules exerting similar CD16-oriented regulation might be found in the future. The lead-protein principle was confirmed for tumour cells (6) and both, the clinical confirmation in ALS (62,67) and its experimental proof-of-principle in tumour cells (6), are now new drivers in other fields when it comes to tailor toponome-target based therapies, e.g., in cancer.

For ALS the impact of ICM has been to find an ALS toponome and a therapy method exerting direct or indirect regulation of the lead-protein CD16 (64–66), now confirmed clinically (62,67). The derived immediate advantage of this ICM approach is early diagnosis in order to prevent disease progression: ALS is often difficult to diagnose in early stages, when patients complain, for example, of a clumsiness in their motor neuron (63) as part of the overall CD16 motif (Fig. 3) (64–66) are critical aberrant cells because they invade and actively damage the neurons in the motor system (to be published). These cells can be interpreted as aberrant T cells (not monocytes) expressing that motif as a biomarker of their agenda. When the cells published in 1995 (63) were mapped for the ALS toponome (Fig. 3) we have never seen these cellular motifs in other contexts, as revealed by imaging of normal controls (n = 8), and disease controls (stroke, n = 3; polymyositis, n = 6), but have seen them regularly in ALS with supranuclear palsy or predominant involvement of the upper motor neuron (n = 21) (63–66). Together these data suggest presence of an abnormally differentiated cellular immune system driving these cells into aberrant uncontrollable homing to the CNS.

Lessons

Lessons learned from the clinically and experimentally validated ICM based predictions are: Tissues must be intact for successful discovery of topologically intact molecular systems and for this purpose must be fixed as gently as possible (6,9); Isolation of tissue components (cells or molecules) would disrupt the topological properties of the system as a whole; isolated cells do not reveal the system, since disease-driving molecular systems are robust structures involving many cells on a multi-molecular scale in tissues ranging from subcellular to transcellular dimensions (Figs. 1–3). This shows that he best ‘biochip’ is the tissue itself (including blood as fluid tissue). Topologically ordered states in health and disease are keys to assess (dys)function with certainty. If relevant tissues are subjected to high-dimensional ICM with a PCMD ranging from 218 to 256100, or 65,536 100 per data point, then the key information on the spatial and functional coding of disease-driving and normal networks will be automatically extracted and (most importantly) found in a hypothesis free manner. Such data will often contain relevant lead proteins as we have established by studying cell surface associated molecular systems (6,17,64–66). Lead proteins controlling disease-driving molecular networks can be mathematically described as combinatorial molecular motifs (2,6). They can be interpreted as robustness nodes of disease, because blocking or inhibiting the lead proteins detected by ICM leads to disassembly of the pathogenic network and loss of (dys)function as shown for tumour cells experimentally (6) and now for ALS clinically by successfully downregulating the ICM predicted ALS lead-protein CD16 (62). This shows, that specific robustness nodes present in tissue, are built by spatial molecular networks with unique topological coordinates.

Implications for Drug Discovery and Future Strategies in ALS Research

The pharmaceutical industry and the healthcare system are facing major challenges because of high failure rates of drugs, as reported, e.g., for cancer (“High attrition rates—where are we going wrong?” (69). The frequently quoted $1 billion to bring a new drug to market has risen to $4 to 5 billion by some estimates (www.forbes.com/sites/matthewerper/2012/02/10/the-truly-staggering-cost-of-inventing-
new-drugs). To address this gap, the U.S. President’s Council of Advisors on Science and Technology (PCAST) published a report in September 2012 on propelling innovation in the pharmaceutical industry, which commented that “the ecosystem for public health is under significant stress” and that research and development (R&D) productivity is declining [commented in Ref. (70)]. Inefficient target discovery is likely to be one of the problems (69). The current, above briefly described experience and proof of concept with ICM based drug discovery suggests that future strategies must include the discovery of disease mechanisms in situ on a large scale of in situ protein networks to improve efficiency of drug target prediction, as shown for ALS. Safety assessment is another candidate for the ICM-toponome approach. Technologically, today a complete decoding of all protein networks (the e.g., approx. 6,000 cell surface associated druggable biomolecules in tissues) is feasible by applying a net of ICM robots, for example as a globally accessible platform in appropriate centers. Concerning ALS, the specific advantage of ICM based toponome approach lies in the technical feasibility to monitor PBMCs for presence of the ALS motif (Fig. 3) and thereby improve early diagnosis, explore new toponome motifs in disease subsets, monitor the found motifs over time, and potentially initiate early therapeutic targeting of the lead protein(s) to potentially counteract progressive physical signs of the disease. A key for success can be based on ICM-toponome information content by co-mapping many thousand different multi-protein assemblies at a time, because these data contain the disease specific systems functions. Globally accessible ICM platforms will significantly enhance toponome research and drug discovery.

CONCLUSION

Chronic diseases are robust systems whose emergent molecular properties are hardly understood. High drug attrition rates and failures of treatments (69,70) show that these systems have not yet been therapeutically targeted at their robustness nodes. Direct ICM imaging of disease protein networks inside the diseased tissue provides a new option for therapeutic success, because (i) the hierarchy of molecular disease networks with lead proteins can be directly visualised and quantified inside the relevant tissue (6), (ii) these networks can be disassembled by inhibiting the lead protein controlling the network topology and (dys)function (6), as shown experimentally, and (iii) ICM based prediction and downregulation of the lead protein and/or functions of it in ALS leads to therapeutic effects, as shown in a clinical trial (62). This may indicate that the molecular disease robustness mechanisms in ALS can be broken, suggesting that the ICM approach is likely to provide similar access to the robustness nodes in other chronic diseases. In ALS, early diagnosis and treatment can now be facilitated by ICM-PBMC screening for the ALS toponome.

ACKNOWLEDGMENTS

The author thank ToposNomos Ltd. for providing access to the Imaging Cycler® reference lab and Andreas Krusche and Reyk Hillert for helping with the figures and formatting the manuscript.

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