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Unlocking Single-Cell Immunometabolism Research: A Call for Accessible Tools

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54 Recent technological advances permit the profiling of metabolic changes in single cells, shedding light
55 on how metabolism regulates immune responses. We advocate that accessible and standardized tools
56 are needed to reduce the barrier of entry to immunometabolism studies and facilitate the translation
57 of fundamental findings towards clinical applications.
58

59 Immunometabolism: from niche to research field

60 Historically considered as a set of passive house-keeping functions required for cell survival,
61 metabolism is now recognized to play an active and multifaceted role in regulating immune cell fate,
62 function and disease. However, progress in the field of immunometabolism has been hampered by the
63 low sensitivity of metabolic profiling approaches. The enormous diversity in the structure and
64 biochemical properties of metabolites makes it difficult to detect metabolic changes in rare cell types.
65 This issue is further complicated by compound fragility and limited methods to amplify signal, as is
66 common for nucleic acid-based profiling methodologies, complicating translational research on cells
67 isolated from patient samples, which are often too scarce or too heterogeneous to perform
68 “traditional” metabolic analyses. To address this issue, the immunometabolism field has focused on
69 developing high-sensitivity and low-input assays that can profile small amounts of starting material,
70 even with single cell resolution.
71

72 Key developments in recent years include cytometry-based techniques that can quantify enzyme and
73 transporter expression levels (single-cell metabolic profiling [scMEP]¹; assessing Metabolism by Flow
74 cytometry [Met-Flow]²), nutrient uptake assays (e.g. QUAS-R)³ and metabolic pathway capacities and
75 dependencies (Single-cell Profiling and Imaging of Cell Energy Metabolism [SPICE-Met]⁴; Single Cell
76 Energetic metabolism by profiling Translation inhibition [SCENITHTM]⁵). In parallel, single-cell
77 metabolomics, fluxomics and mass spectrometry imaging (MSI) are rapidly improving in sensitivity and
78 throughput, permitting direct metabolite quantification in rare cell types and tissues^{6, 7} down to the
79 single-cell level (SpaceM)⁸ (summarized in Supplementary **Figure 1, top panel**). Furthermore,
80 antibodies against specific metabolites are now available via immusmol and Immundiagnostik AG,
81 allowing for the detection and quantification of metabolites in samples with limited material. As
82 technologies mature, they are being increasingly integrated with multi-omics, imaging and lineage
83 tracing assays – a comprehensive approach that adds spatial and functional context to metabolic
84 measurements across cells, tissues and organisms⁹. Importantly, many of the innovations detailed
85 above have been fueled by an expanding catalogue of reagents including chemical probes and dyes,
86 antibodies, and pharmacological inhibitors as well as by increases in mass spectrometry sensitivity,
87 and by computational methods and software for data integration and interpretation. This
88 development illustrates the importance of an interdisciplinary approach that allocates the collective
89 resources of the industry and public research sectors in an efficient way to advance the field. Dramatic
90 innovations have helped the immunometabolism field to mature and advance our fundamental
91 understanding of immunity and inflammation, yielding a number of promising translational research
92 innovations, from the refinement of cell culture systems to metabolite derivatives that suppress
93 immune cell activity¹⁰ and the optimization of cell therapies¹¹. To obtain biologically and metabolically
94 meaningful data, a selection of tools should be utilized in a complementary fashion since all
95 immunometabolic profiling approaches have their advantages and disadvantages. For a
96 comprehensive overview of the limitations of current methods, as well as selection guides and other
97 summarizing tables, we refer the reader to recent articles on these subjects^{7, 12, 13}.
98

99 Here, we highlight a key challenge in immunometabolism research and provide guidance on how to
100 overcome it to advance the field. One major problem is that immunometabolic approaches typically
101 require significant expertise and resources to establish and implement existing assays, and to analyze
102 and interpret the associated data. Considerable resource investment and repeated trial-and-error
103 processes are thus required to optimize and adapt state of the art immunometabolism approaches to
104 different research contexts. A major challenge will thus be not only to extend, but to simplify,

105 generalize and standardize the existing toolkits, and to encourage companies to develop and offer a
106 range of readily accessible optimized tools and reagents. Over the longer term, these tools will enable
107 the scientific community to perform immunometabolic assays in a more rigorous and reproducible
108 manner, which is critical for translating immunometabolism approaches into clinical practice.

110 **Resources needed to advance immunometabolism research**

112 *The future of single-cell metabolic analyses*

113
114 As outlined above, a series of novel immunometabolic methods use antibodies to quantify metabolic
115 cell states. Currently, most available commercial antibodies directed against enzymes or regulators
116 involved in the control of metabolism are unconjugated and thus requires custom coupling, a step that
117 is time- and resource-consuming, error-prone and adds another source of variability that could limit
118 reproducibility. In addition, some metabolic pathways cannot be easily assessed due to the absence of
119 cytometry-validated antibodies targeting associated enzymes and transporters. We thus advocate for
120 the development of commercially available validated antibodies and calibrated ready-to-use
121 cytometry and imaging panels that would allow the inclusion of antibody-based metabolic profiling
122 approaches in the common toolbox of immunometabolism research. Here, we propose three panels
123 of enzymes that can be used alone or in combination to gather insights into glycolysis, mitochondrial
124 respiration (panel 1 A and B), and other metabolic pathways (panel 2) in single cells using fluorescence-
125 or TOF (time-of-flight)-based cytometry and imaging (**Table 1**).

126
127 New analytical tools and approaches are required to accurately capture the spatiotemporal dynamics
128 of immunometabolic changes. With the emergence of techniques like mass spectrometry imaging,
129 imaging mass cytometry (IMC), multiplexed ion beam imaging (MIBI), and *in situ* metabolic
130 measurements, researchers can now spatially map the metabolic configuration of immune cells in
131 tissues. Yet, defining the kinetics by which metabolic rewiring occurs remains a challenge. Capturing
132 this temporal aspect of immunometabolism will facilitate the rational design of therapies by separating
133 metabolic drivers of disease and immunity from bystander effects. In general, mass spectrometry and
134 other analytical tools with a higher sensitivity will enable capturing measurements with improved
135 spatial resolution from low input material facilitate the study of rare cell populations with better
136 precision. Moreover, mass spectrometry can also provide extra layers of information about the
137 subcellular distribution and organization of metabolic events.

138
139 Another challenge is the development of alternatives to fluorescently labeled metabolites, such as 2-
140 NBDG, to measure glucose flux. CLICK chemistry-based approaches^{3, 14} that have been developed to
141 measure metabolism provide one potential solution. However, directly measuring enzymatic activities
142 by flow cytometry would allow for more functional metabolic analyses at single-cell level. Different
143 sensors (e.g. for NAD⁺/NADH, lactate, etc¹⁵) to quantify metabolism using imaging and cytometry-
144 compatible fluorescence readouts are emerging. Incorporating these sensors into commercially
145 available lentiviral backbones and/or mouse models should improve the accessibility of such methods.
146 Other genetic tools that will advance immunometabolism research include functional genomics
147 approaches such as Perturb-seq that can functionally test the importance of metabolic genes in
148 regulating immunity and disease outcome. Finally, there is a need for mass spectrometry approaches
149 for isotope tracing and fluxomics at the single-cell level that would allow the unbiased and quantitative
150 profiling of metabolic activities of glycolysis and other metabolic pathways. New technologies enabling
151 these steps are currently under development (**Figure S1, bottom**). In addition to developing new tools,
152 both academia and industry should focus on the refinement of existing tools that have been recently
153 developed to reduce technological barriers for researchers entering the field.

155 *Standardized analysis approaches*

157 In parallel with experimental advances, new computational and analytical tools are needed to analyze
158 multi-modal immunometabolism data. Single-cell omics data is characterized by high levels of sparsity
159 (i.e. limited depth of coverage), variability and noise. Furthermore, due to the non-linear relationship
160 between RNA, protein and metabolite levels, it is non-trivial to consolidate data across different omics
161 modalities and to link metabolite levels and RNA/protein expression information to cell function. In
162 addition, different assays measure different aspects of metabolism, for example enzyme expression
163 patterns versus metabolic fluxes. There are no commonly accepted ways to visualize multimodal
164 metabolism data in a single plot. Significant computational biology expertise is therefore needed to
165 consolidate metabolomics measurements across modalities into meaningful insights. Some
166 bioinformatics pipelines to analyze multi-omics data have been developed, for example pairing protein
167 and RNA measurements using the Seurat analysis package, but such tools are not tailored to study
168 metabolic processes.

169
170 Extracellular flux analysis using the Seahorse device helped to open up and grow the
171 immunometabolism field not only by offering new tools, but by providing analysis pipelines to easily
172 obtain glycolysis- and respiration-related metabolic parameters. In a similar way, SCENITH™ allows
173 calculating metabolic dependencies of immune subsets in a user-friendly manner⁵. Hartmann et al.
174 calculate glycolysis and respiration scores by taking the expression of metabolic enzymes in the
175 respective pathways into account¹. Furthermore, SpaceM and other mass spectrometry-based
176 approaches demonstrate the feasibility of direct metabolic profiling⁸. Integrating metabolic analyses
177 and visualizing metabolic perturbations into existing, and potentially also novel, software will help
178 establish immunometabolic profiling as a common research practice. In this context, having user-
179 friendly tools to infer metabolic information from scRNA-seq and CRISPR screens will be useful to add
180 an immunometabolic perspective on such data. Currently, most existing analysis pipelines are
181 implemented as programming scripts that are not generalizable and, therefore, cannot analyse
182 different data types depending on the context of research questions. Moreover, public sharing of
183 generated data is crucial to provide reference datasets and help develop software tools. Defining data
184 and metadata standards and consolidating existing analysis approaches into an open-source software
185 package, web portals and applications, such as has been done for single-cell RNA sequencing analysis,
186 is a necessary step to further advance the immunometabolism field.

187 188 *A wish list for immunometabolism research*

189
190 To increase accessibility, standardization, interpretation and general use of current tools by a broader
191 audience, we here summarize key aspects into a wish list:

- 192 • Standardized ready-to-use antibody panels targeting a common set of metabolic regulators
193 for immunometabolism profiling (proposed in Table 1)
- 194 • Validated and conjugated monoclonal antibodies to customize cytometry and histology
195 metabolic panels
- 196 • Readily available SCENITH™ kit containing standardized components
- 197 • New mass spectrometers and commercial mass spectrometry services capable of profiling
198 single cells or populations of relatively rare cells (< 10,000 cells)
- 199 • Software to analyze and visualize datasets from the distinct immunometabolic profiling
200 approaches
- 201 • Incorporate genetically encoded biosensors for metabolic pathways into vectors and mouse
202 models (e.g. SPICY mice⁴)
- 203 • Click chemistry-based approaches enabling to assess nutrient uptake and metabolic
204 dependencies of single cells

205
206
207

208 **Conclusion**

209

210 As the immunometabolism field is rapidly evolving, we must consider how to make technological
211 innovations broadly accessible to bridge the gap between research and clinical practice. With this
212 commentary, our goal is to advocate for the development and commercialization of versatile,
213 accessible, and standardized tools allowing the community to take a crucial step forward and unlock
214 single-cell immunometabolism research. We urge both researchers and companies to respond to our
215 call to collaboratively propel the immunometabolism field forward.

216

217

Table 1. Proposed panels for cytometry and imaging-based single-cell profiling

1. Glycolysis & respiration panels		Pathway	
A. Basic			
GLUT1	Glucose uptake	<i>glycolysis / fermentation</i>	
PKM	Glycolysis / transcriptional regulator		
LDH	Pyruvate to lactate conversion		
CS	Citrate synthase	<i>TCA cycle / mitochondrial respiration</i>	
IDH	Isocitrate dehydrogenase		
ATP5A	ATP synthase / ETC complex V		
B. Extension *			
HK	First glycolytic enzyme	<i>glycolysis / fermentation</i>	
GAPDH	Glycolytic enzyme / immune regulator		
MCT1	Lactate transport		
OGDH	alpha-ketoglutarate dehydrogenase	<i>TCA cycle / mitochondrial respiration</i>	
SDH	Succinate dehydrogenase / ETC complex II		
CytC	Cytochrome C, essential for ETC function		
2. Broad metabolism panel **			
G6PD	G6P dehydrogenase, rate-limiting PPP step	<i>Pentose phosphate pathway</i>	
CD98/LAT1	Essential amino acid transporter	<i>Amino acid metabolism</i>	
ASCT2	Alanine/Serine/Cysteine-preferring amino acid transporter 2		
GLS	Glutamine to glutamate conversion		
CD36/FAT	Fatty acid transporter	<i>Fatty acid metabolism</i>	
CPT1A	Fatty acid shuttling into mitochondria		
ACLY(-P)	Citrate to acetyl-CoA conversion for fatty acid and cholesterol synthesis		
FASN	Fatty acid synthase		
ACC / ACAC	Acetyl-CoA carboxylase / Fatty acid synthesis		
HIF1A	Hypoxia & inflammation-induced transcription factor		<i>Metabolic regulation</i>

* extension of basic glycolysis/respiration panel for more detailed metabolic insight

** extensive metabolic panel to be used in combination with the glycolysis & respiration panel A. (and B., if so desired)

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289

290 **Conflict of interest**

291 R.J.A. is main inventor of SCENITH™ (Patent PCT/EP2020/060486) and in the process of licensing the
292 technology for commercialization of kits and providing patient samples analysis services. In the past three
293 years, C.A.L. has consulted for Astellas Pharmaceuticals, Odyssey Therapeutics, Third Rock Ventures, and T-
294 Knife Therapeutics, and is an inventor on patents pertaining to Kras regulated metabolic pathways, redox
295 control pathways in pancreatic cancer, and targeting the GOT1-ME1 pathway as a therapeutic approach (US
296 Patent No: 2015126580-A1, 05/07/2015; US Patent No: 20190136238, 05/09/2019; International Patent No:
297 WO2013177426-A2, 04/23/2015). T.A. holds several patents focused on imaging mass spectrometry and leads
298 a startup on single-cell metabolomics incubated at the BioInnovation Institute. CO holds several patents on
299 AHR activation and tryptophan catabolism and is founder and managing directors of cAHRmeleon Bioscience
300 GmbH. The other authors declare no conflicts of interest.

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