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Parhelia Omni-Stainer™ revolutionizes the IMC workflow, producing astonishing staining quality on the first try

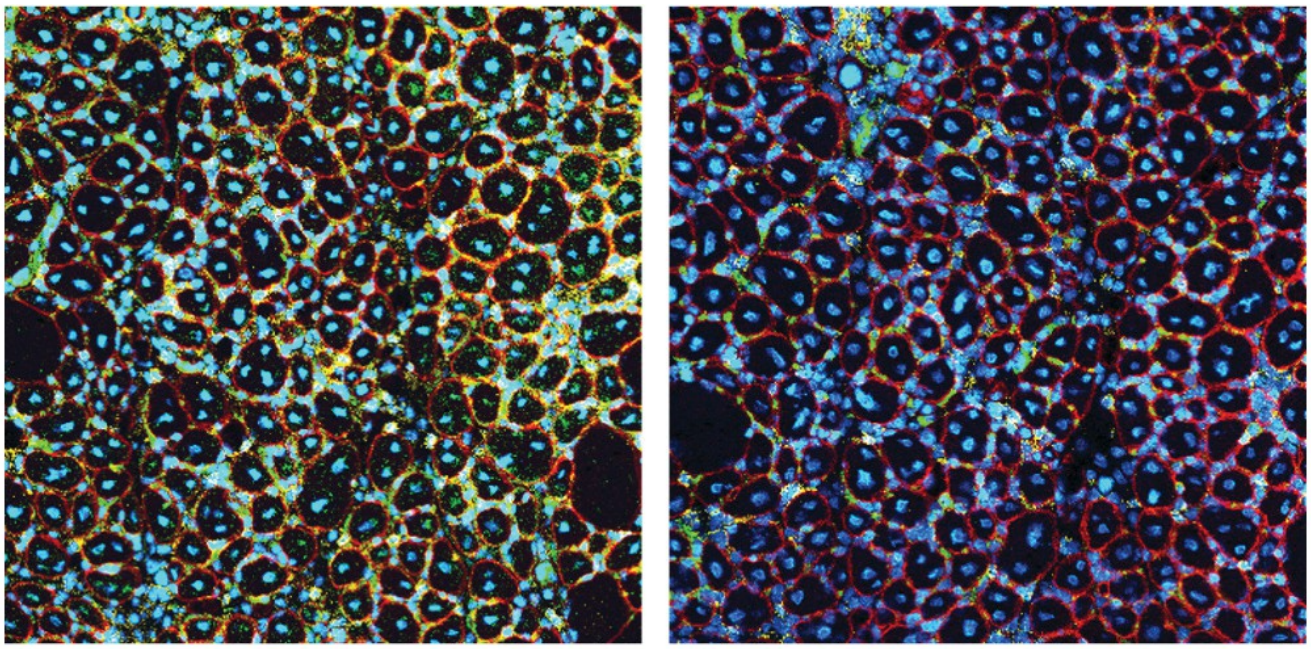
by Nels Wedin and Nikolay Samusik, Parhelia Biosciences, 2023

Summary: Porpiglia Lab verifies Parhelia Omni-Stainer™ for Imaging Mass Cytometry (IMC) sample prep and compares it side-by-side with the customary Hydrophobic Barrier Pen (HBP) workflow. In their experience, the Omni-Stainer™ system simplifies sample handling, saves precious reagents, and **produces superior-quality staining results.**

IMC IMAGE OF MOUSE SKELETAL MUSCLE

Hydrophobic Barrier Pen

Parhelia Omni-Stainer™



Laminin DNA CD31 CD44 CD45

At the Aarhus University of Denmark, a team headed by Prof. Ermelinda Porpiglia employs novel single-cell technolo gies, such as single-cell mass cytometry (CyTOF) and IMC to study the relationships between muscle stem cells and immune cells in aged and dystrophic skeletal muscle. Specifically, their research focuses on understanding how unique interactions between immune cells and muscle stem cells orchestrate effective muscle regeneration and on identifying the cellular interactions and signaling pathways that are impaired during ineffective muscle regeneration, aging, and muscular dystrophy.

Postdoctoral researcher Kedar Ghimire is taking the lead in developing an IMC assay to image murine skeletal muscle samples across the time series of muscle regeneration after injury. IMC was conducted at Aarhus University on the Standard BioTools' Hyperion instrument (Alexander Schmitz, mass cytometry unit). The advanced multiplexing nature of IMC provides him with valuable information about progressive changes in tissue morphology throughout the regeneration process while allowing precise measurement of expression levels across dozens of markers and quantification of spatial interactions between multiple key players, such as muscle fibers, muscle stem cells, vasculature, and various immune cell subsets.

The researchers hope that the quantitative spatial phenotype information will help elicit novel strategies for boosting muscle tissue repair via immune system modulation. Ultimately, the goal is to find new ways to help patients with various muscle degeneration disorders.

There is one catch, however**before a tissue specimen can be analyzed on the (Hyperion) IMC instrument, it needs to undergo a tedious 2.5-day sample prep (aka staining), which involves consecutive incubation of the tissues with an array of solutions:** blocking buffers, metal-conjugated antibody cocktails, alcohol, metal-based nuclear stain, water, etc. The standard implementation of this protocol involves mounting the sample on a microscopy slide, encircling it with a Hydrophobic Barrier Pen (HBP), and then using a handheld pipette to carefully apply and remove consecutive staining reagents, while precisely timing the incubations in between. Kedar writes, *"For a scientist like me with an experience in cell and tissue staining, one of the most irritating parts has been to stain the cell/tissue of interest by creating a boundary around it with a hydrophobic barrier pen and hope that the boundary holds throughout the staining period without any leaks during day or in worst case, during overnight antibody incubation. Not to mention the contamination of [the] barrier chemical into the tissue staining area itself. In addition, moving several slides around when necessary was difficult since I had to focus on ensuring that the liquid within the boundary does not spill while moving. This gets from bad to worse if you are staining many slides and not just one. If just one slide spills, you lose time to recreate [the] boundary and it messes up the workflow and timing. I always wondered if there was a solution to all of this? I came to know recently, there is one! Using a Parhelia staining system, I could use less antibodies, buffers, stain several slides in a single chamber..and the best part, I could finally kiss goodbye to using hydrophobic pen."*

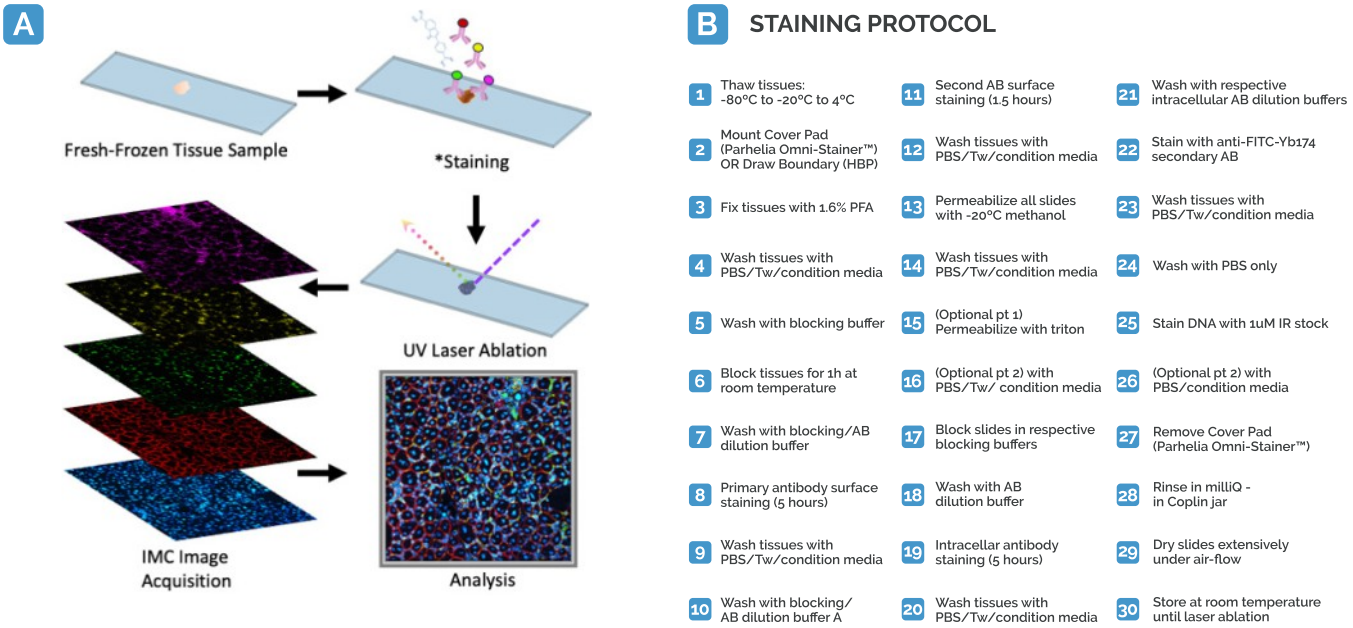


Figure 1: (A) IMC workflow shared for Parhelia Bio Omni-Stainer™ S12 Module and HBP, and **(B)** the 2.5-day staining protocol evaluated on both sample prep technologies. AB=Antibody.

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HBP-based staining is simple, but not easy; mastering it requires a great deal of hands-on attention, fine motor skills, and plenty of time to practice. There are numerous well-known pitfalls including a high risk of sample damage, reagents drying onto the sample in between the removal and the application, and, most notoriously, certain staining reagents being prone to running over the barrier, especially solutions that contain alcohols or detergents.

Frustrated with the HBP technique, Porpiglia Lab decided to use Parhelia Bioscience's Omni-Stainer™ S12 Module, which utilizes the capillary principle within a laminar flow environment. It operates by immersing the entire sample in a thin liquid-filled chamber, which is formed by sandwiching the tissue-holding slide with a specially designed Cover Pad. This Cover Pad is held above the tissue by thin adhesive spacers.. The chamber rests at a 45˚ angle within the Parhelia Omni-Stainer™ instrument, and the staining solution is securely held in place by the capillary force during the incubations. When the incubation step is over, the old solution is gently displaced by simply dispensing a pre-measured amount of a new solution onto the top edge of the chamber. Gravity and capillary force do the rest. Thus **pretty much any staining protocol can be implemented either manually or automatically via robotic liquid handling.**

Kedar Ghimire took on the task of thoroughly comparing the efficiency and reliability of both systems, to which end he performed a series of experiments where he stained Optimal Cutting Temperature (OCT)-sectioned murine skeletal tissue samples side-by-side using HBP and the Parhelia Omni-Stainer™ S12 Module.

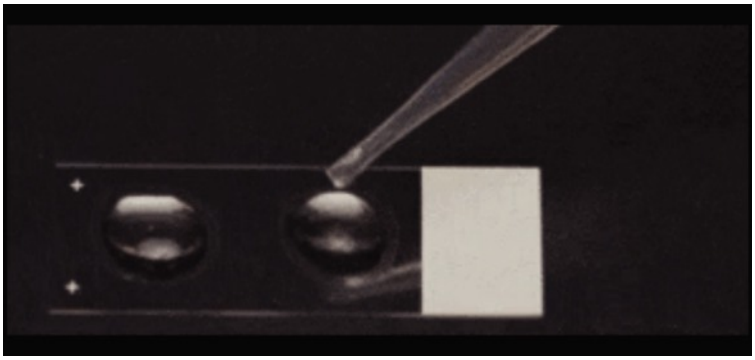

Figure 2: Hydrophobic Barrier Pen operation

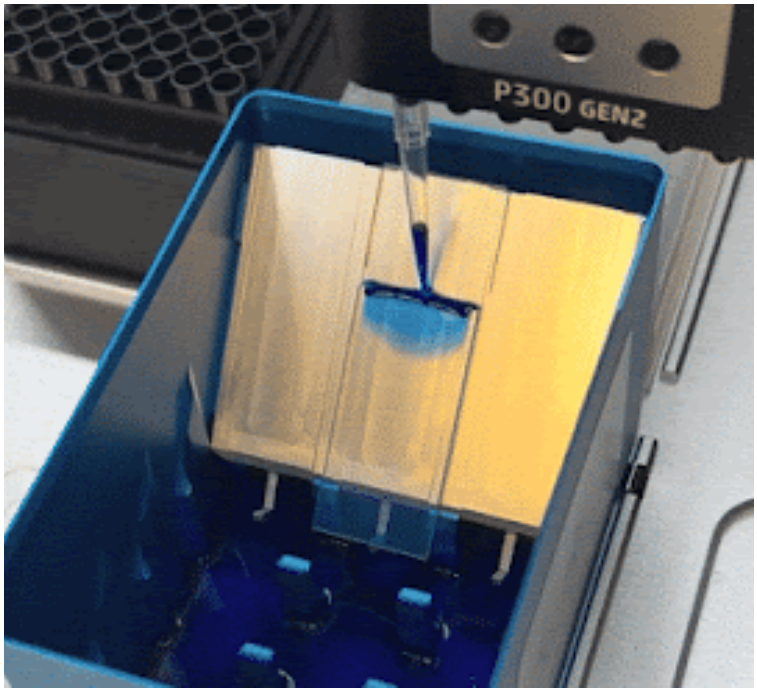

Figure 3: Parhelia Omni-Stainer™ S12 Module operation

Workflow Stability and Ease of Handling

While manually staining samples side-by-side on Parhelia Omni-Stainer™ and the conventional HBP setup, Ghimire observed an immediate difference in the stability of the two systems. The Parhelia Omni-Stainer ™, once set up, was stable throughout the long-lasting staining protocol, showing no leaks or disturbances. Conversely, as the protocol advanced, the conventional workflow showed the usual drawbacks –leaks forming over the 'thin spots' in the pen boundary. Ghimire states, *"This [propensity to leakage] was particularly evident during our permeabilization step where we use methanol. During this step, we had to constantly add extra methanol as it would leak out from the boundary onto the entire slide. We thus had to re-draw the Hydrophobic barrier pen boundary several times, which increased the risk of disturbing the tissue."*

In terms of sample handling, Parhelia Omni-Stainer™'s self-contained capillary chambers eliminated the need to touch the slides and minimized the risk of sample damage, reagent spillage, and drying - thus improving the overall robustness of the staining protocol.

Minimizing Reagent Loss

IMC sample staining relies on special metal-conjugated antibodies, which are generally costly, and their production involves global sourcing of high-purity heavy isotopes that are vulnerable to supply chain disruptions. Researchers who routinely run IMC assays are keen on using these precious reagents efficiently.

The barrier pen technique had been created to minimize the reagent use by confining the liquids to the tissue-occupied area of the slide. In practice, however, covering even a medium-sized tissue section with this technique requires 150 - 200 µl of antibody solution, which in the case of a typical IMC panel translates into a cost of hundreds of dollars per sample. And when staining large samples and TMAs, covering the entire sample area may require up to 400 µl of the antibody solution, proportionally driving the cost.

Ghimire highlights that, in addition to its user-friendly nature, ***"the major advantage of the Parhelia system is the low volume required during staining."*** In this experiment, the Parhelia Omni-Stainer™ only required 100 µl to stain an entire 75x25 mm (18.75 cm2) slide, while the HBP protocol used 100-150 µl to stain an HBP boundary of approximately 4 cm2. Therefore, Parhelia Omni-Stainer™ S12 Module provides a stainable area 4.6875 times larger than the HBP, while utilizing the same or even less volume of reagents. That volume is stable and predictable irrespective of the tissue size, which in practical terms allows for a further increase in the throughput by positioning several samples side-by-side on one slide while staining them under the same Cover Pad.

Image Quality

Across multiple markers, the samples stained with **Parhelia Omni-Stainer™ showed greater specificity and signal-to-noise ratio than the HBP-stained ones** (Figure 4). This was especially pronounced for CD31 and CD44 (Figure 4), where HBP staining showed high levels of non-specific binding in the nucleus and on cell membranes.

The positive signals were generally comparable between the two systems, except for the nuclear and laminin staining, which appeared weaker in the case of the Parhelia system. This was a surprising finding that Parhelia's team hasn't observed with other protocols and certainly warrants a follow-up investigation, e.g., experimenting with increased staining reagent concentration. However, one unique feature of the IMC-TOF instruments is a particular type of channel spillover, whereby strong signals on certain channels may create 'ghost images' in neighboring channels. Therefore, IMC researchers generally prefer having weaker but cleaner signals and often 'titrate down' antibodies to bring their positive signal into the 'sweet teens' range and thus minimize the spillover effect.

Therefore, attenuated and cleaner signals observed with the Omni-Stainer™ instrument may be preferable for all intents and purposes of the IMC workflow.

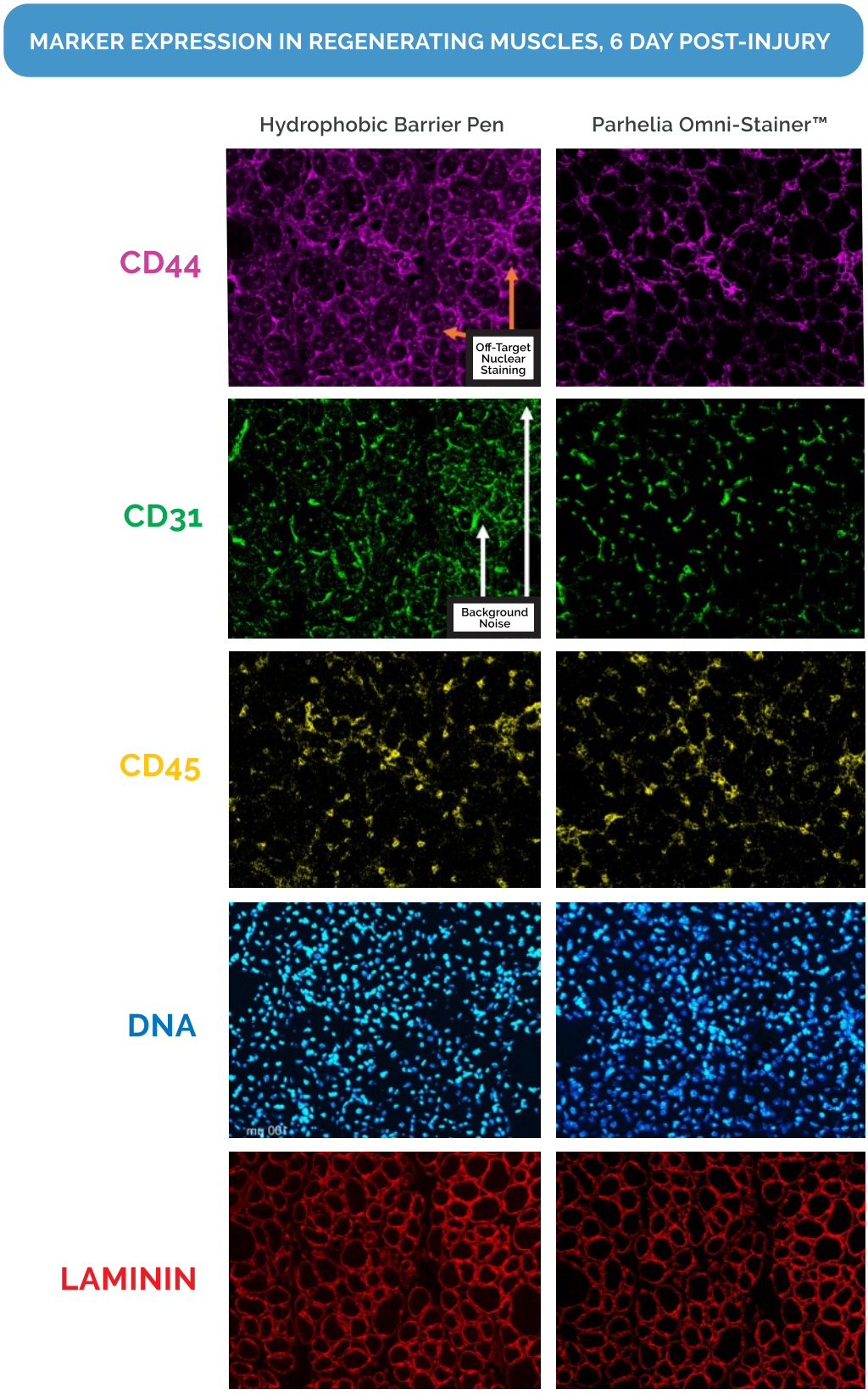

Figure 4, IMC staining method comparison (HBP vs. Parhelia Bio Omni-Stainer™ S12 Module) for regenerating skeletal muscle tissue, surface staining (CD31, CD45, CD44, Lami - nin) was performed before permeabilization with MeOH and Tween, used for intracellular staining. Source: Porpiglia Lab, *unpublished.*

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A visual comparison of the merged images (Figure 5) is particularly interesting: the accumulation of the background across all channels creates a visual effect where most cell membranes in the HBP-stained image end up being colored in various 'shades of the rainbow' (blended marker signals), making it visually hard to distinguish specific cell types. By comparison, the relatively low background in the Parhelia-stained sample allows for immediate visual discrimination of muscle fibers, nuclei, blood vessels, and immune cells, as they each appear marked with a distinct color (red, blue, green, and yellow, respectively), allowing the researchers to start analyzing interactions between those key players.

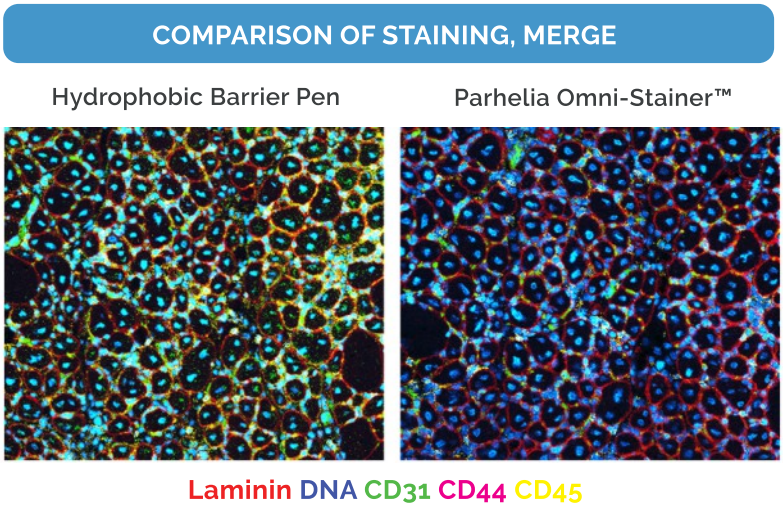

Figure 5, IMC staining method comparison on post-injury skeletal muscle tissue; images show merged co-detection of CD31, CD44, CD45, and laminin protein markers with DNA - Source: Porpiglia Lab, *unpublished.*

	HYDROPHOBIC BARRIER PEN	PARHELIA OMNI-STAINER™
Stability	Low	High
Handling	Hands-on	Hands-off
Reagent Usage	Variable: 100-400µl depending on the sample size	Consistent: 100µl any size sample
Leakage	High Risk	Low Risk
Drying	Less Efficient	More Efficient
Signal-to-Noise Ratio	Lower	Higher
Specificity	Variable	High
Background Noise	High	Low

Conclusion

Porpiglia Lab's team successfully implemented the entire IMC sample prep workflow on the Parhelia Omni-StainerTM system and performed a comprehensive side-by-side comparison against the state-of-the-art HBP-based workflow. They found the Parhelia system to be superior in all aspects during this experiment, including stability, handling, reagent use, and image quality and decided to employ the Omni-StainerTM system workflow for all their future IMC stains. They recently stained an entire time course of an acute injury on a single slide and then obtained high-quality data. They can clearly identify dramatic immune infiltrates at day 3 post-injury among the degenerating muscle fibers and distinct immune niches that arise 6 days post-injury in the proximity of centrally nucleated, regenerating fibers (Figure 6). Importantly, the ability to stain an entire time course on a single slide provides the advantage of being able to accurately compare signal intensity across different time points without having to worry about technical variability.

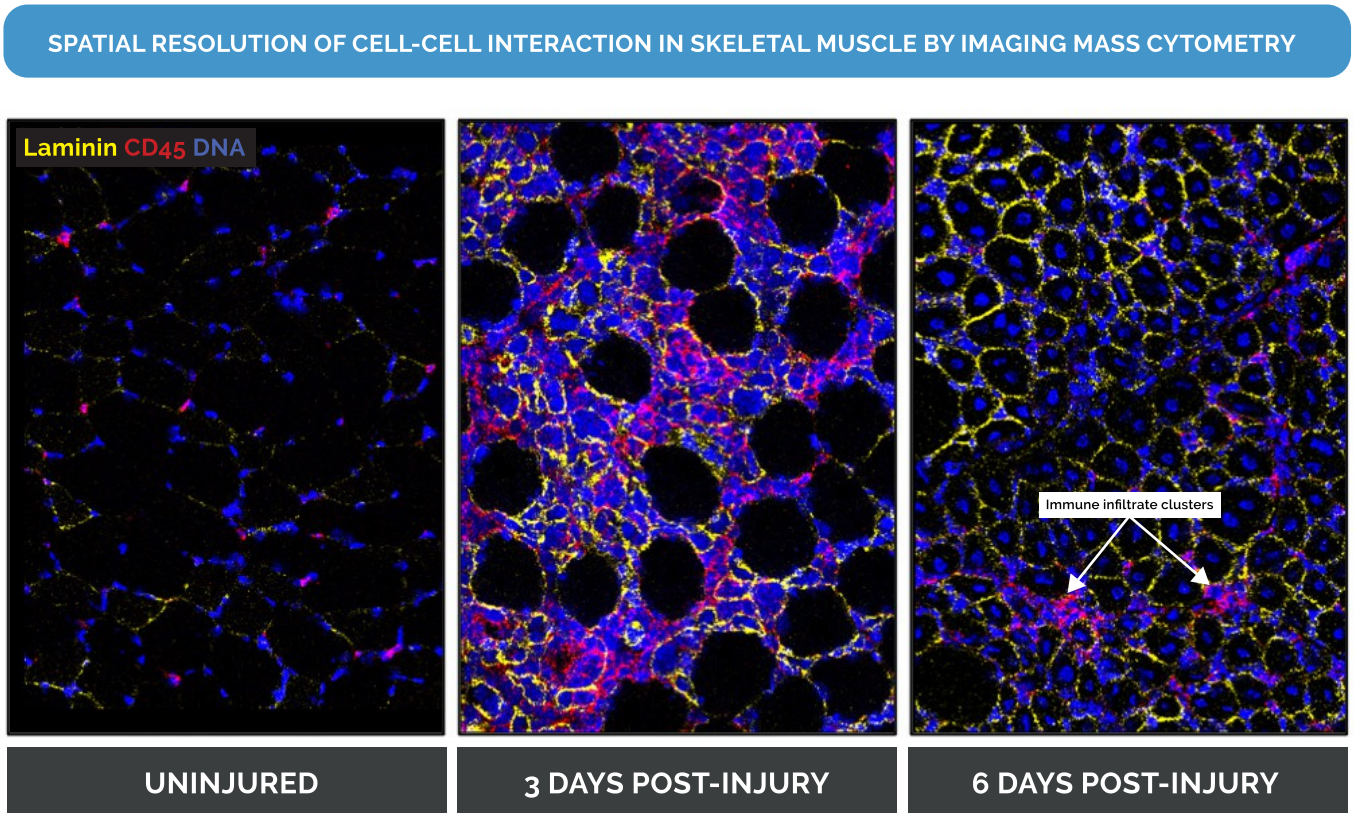

Figure 6, Spatial Cellular Resolution of Skeletal Muscle Regeneration by IMC; the uninjured muscle shows a normal distribution of cells. The day 3 image reveals a dramatic influx of immune cells (CD45, red) among small degenerating muscle fibers, indicating an active immune response. By day 6, distinct immune cell niches are seen near the centrally nucleated regenerating fibers, highlighting a shift towards tissue regeneration. The samples were stained using Parhelia Omni-Stainer™.

In the current study, the Parhelia Omni-Stainer™ was operated manually, which in and of itself turned out to be very beneficial. In addition, the lab has been able to gain access to an Opentrons OT-2 robot in a facility next door. Together with the Parhelia Apps Team, they plan to convert the IMC staining protocol into a simplified OT-2 Python script, hoping to fully automate the tedious 2.5-day process and also improve staining consistency and reproducibility.

Keep an eye out for our follow-up study, as we delve into full automation for enhanced efficiency and precision!

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