Supplementary Material and Methods:

MICS technology and immunohistochemistry were performed on bone biopsies fixed in 10% neutral buffered formalin and decalcified with EDTA (12.5%, pH 7.0) from 15 hours. Tissue identity and integrity were rigorously validated in collaboration with board-certified pathologists to ensure the accuracy and quality of the samples by Hematoxylin and Eosin (H&E) staining. Slides preparation and sections of 4 μm were cut according to the MACSwell™ Imaging Frames indications. Antibodies supplied by Miltenyi Biotec, annotated as pre-tested for MACSima Imaging Cyclic Staining (MICS), were used at the recommended dilution specified by the provider. Miltenyi Biotec determines this dilution through titration experiments on positive control tissue sections with known target expression. The antibody conjugate concentration yielding specific tissue staining with the highest signal-to-background ratio is selected as the recommended dilution. If required, the dilution was adjusted to account for differences in target expression across tissue samples.

For immunohistochemistry tissues were embedded in paraffin, and sections were deparaffinized, rehydrated through graded alcohols, and subjected to antigen retrieval. Staining was performed with isoform-specific antibodies against CD38 (Cat. NCL-L-CD38-290by Leica Biosystems), BCL-2 (Cat. NCL-bcl-2 by Leica Biosystems) and Ki 67 (Cat. PA0118 by Leica Biosystems) and binding was visualized using ACE as a chromogenic substrate.

After deparaffinization and rehydration, the BM biopsy slides were placed in a pressure cooker in 0.01M citrate buffer (pH 6.0) and heated for 7 min. Incubation with antibodies was carried out overnight at 4°C. Detection was performed with the DAKO EnVision system according to the manufacturer's protocol.

A customized and supervised panel of 56 antibodies, targeting markers relevant to MM, including immune cells, progenitors, multiple myeloma antigens, plasma cells (PCs), adhesion molecules (CAMs), and ectopic surface markers for TME, were developed. To perform MICS technology (1) either primary antibodies were used in combination with a secondary antibody, or directly coupled to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC) (Supplementary Table 1). This allows for the efficient erasure of signals via photobleaching.

Deparaffinization and rehydration of the tissue slices were performed according to the manufacturer's protocol (Miltenyi Biotec B.V.& Co.KG). In brief, FFPE tissue sections were placed in Xylol (#9713.1; Carl Roth, Karlsruhe, Germany) for 20 min at RT. Deparaffinized tissue was rehydrated through a graded ethanol series (100%, 95%, 80%, 70%, 50%; #100983; Merck, Darmstadt, Germany) and distilled water (1 min each). Following, heat-induced Antigen Retrieval (HAR) with TEC (Tris/EDTA/Citrate)- Buffer pH 9 for 20 min at 98°C was performed.

After cooling down (20 min at 85°C), MACSwell™ Imaging Frames were mounted directly on the tissue slide and covered immediately with an appropriate volume of MACSima™ Running buffer (#130-121-565; Miltenyi Biotec, Bergisch Gladbach, Germany). Then the tissue slices were stained with DAPI solution (a component of MACSima™ Stain Support Kit: #130-127-574) in the dark for 10 min at RT. Finally, tissue slices were washed thrice with an appropriate volume of MACSima™ Running buffer, according to the MACSwell™ Imaging Frame used and were ready to be placed into the MACSima Imaging Platform to start the experiment.

The MACSima Imaging System captured high-resolution images at each staining cycle, producing detailed spatial maps of protein expression within the TME. Before the workflow, regions of interest (ROIs) were defined based on DAPI staining, also in association with H&E staining, to obtain a better region definition. Image processing and analysis were conducted using MACS® iQ View software (Version 1.3.2), which is designed to manage the extensive data generated by the MACSima system.

The workflow included:

i) cell segmentation, performed using advanced tools such as Cellpose and Baysor, converting cell masks into polygons and merging overlapping segments to resolve conflicts.

ii) annotation in which cell types and niches were accurately annotated based on the expression profiles of selected markers, facilitating detailed analysis of the BM TME and EMD sites.

iii) spatial analysis (geometric and spatial statistics were computed to understand different cell types of localization, interaction, and distribution).

Statistical analysis was performed using GraphPad Prism version 10.4.0 (GraphPad Software).

Data from each sample were processed individually in a Python-based analytical pipeline (Python version 3.12.4). Each input CSV file contained matrices representing fluorescence intensity levels assigned to individual nuclei or cells, identified by spatial coordinates (X, Y) and previously segmented by the software.

For each marker, fluorescence intensities were processed using the arcsinh transformation. Data analysis was conducted using the Python package Scanpy (2),

applying default parameters unless otherwise specified. After normalizing the data and scaling expression levels, principal component analysis (PCA) was performed. A detailed inspection of the explained variance plot led to the selection of 20 principal components for downstream analyses, including the computation of neighborhood connectivities (n_neighbors=5). Notably, neighborhood proximity refers to the expression space rather than the physical proximity of cells in the (X, Y) coordinates.

Clustering was performed using the Leiden algorithm (resolution = 0.4, default parameters).

Before assigning cell types to clusters, their spatial distribution was examined. In three EMD samples, clusters (*displaying a grid pattern*) were identified as putative staining artefacts and thus excluded from further analyses.

Cluster annotation was conducted based on the expression levels of known marker genes. Heatmaps were used to visualize these markers, and representative genes for each cluster were identified, ranking genes for characterizing groups with a statistical t-test.

For visualization, two-dimensional Uniform Manifold Approximation and Projection (UMAP) plots were generated using default parameters.

For BM samples, T lymphocytes were virtually sorted to enable a secondary round of clustering based on the variables CD4, CD8a, CD45RA, CD45RO, and CD279. This approach facilitated the identification of CD8 T Naive/Effector, CD8 T Memory, and CD4 T cells. For the monocyte/macrophages population, we could identify M1/M2-like populations based on protein expression profiling as previously published (3).

Following cell type annotation for each sample, we visualized the relative composition of each sample using stacked bar plots and generated spatial distribution images of the identified cell types.

To assess potential cell-cell interactions based on nuclear X, Y coordinates, a neighborhood graph/network was constructed for each sample. In this representation, each cell was considered a node, and an edge was established between two nodes if the Euclidean distance between their respective nuclei was below a predefined threshold of 8μm.

References

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2. Wolf FA, Angerer P, Theis FJ. SCANPY: large-scale single-cell gene expression data analysis. Genome Biol. 2018 Feb 6;19(1):15.
3. Sun J, Corradini S, Azab F, Shokeen M, Muz B, Miari KE, et al. IL-10R inhibition reprograms tumor-associated macrophages and reverses drug resistance in multiple myeloma. Leukemia. 2024 Nov;38(11):2355-2365.

Supplementary Figure Legends

Supplementary Figure 1. Heatmaps of scaled protein expression. For each replicate, heatmaps display cell-to-cell protein expression in bone marrow (BM) samples, grouped by cell type. Used for marker-based cell cluster annotation (Figure 1 D).

Supplementary Figure 2. Heatmaps of scaled protein expression. For each replicate, heatmaps display cell-to-cell protein expression in extramedullary (EMD) samples, grouped by cell type. Used for marker-based cell cluster annotation (Figure 1 H).

Supplementary Figure 3. Uniform manifold approximation and projection (UMAP) illustrates the expression of each marker within bone marrow (BM) and extramedullary (EMD) samples identified via a self-organizing map (SOM).

Supplementary Figure 4. Ultra high-content imaging shows expression of BCL-2 (red), Ki 67 (yellow) and CD38 (light blue) staining with MICS analysis on paired bone marrow (BM) and extramedullary (EMD) sections of all enrolled patients. *CD138 (bright pink) is showed only for EMD#2 to better visualize sample architecture, given the peculiarity of EMD mass, as confirmed by immunohistochemistry (data not shown). Scale bar = 100 µm.

Supplementary Figure 5. Immunohistochemistry validation of CD38, BCL-2 and Ki 67 expression both in bone marrow (BM) and extramedullary (EMD) samples for all enrolled patients.

Supplementary Figure 6. Cell type assortativity in neighbourhood graphs for each bone marrow (BM) and extramedullary (EMD) sample for all enrolled patients. Heatmaps show the fraction of links connecting all possible cell type pairs, indicating cell type mixing.