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Protocol

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An end-to-end workflow for multiplexed image processing and analysis

In the format provided by the authors and unedited

Supplementary Information

Supplementary Note 1

```
## R version 4.3.0 (2023-04-21)
## Platform: x86_64-pc-linux-gnu (64-bit)
## Running under: Ubuntu 22.04.2 LTS
##
## Matrix products: default
## BLAS:   /usr/lib/x86_64-linux-gnu/openblas-pthread/libblas.so.3
## LAPACK: /usr/lib/x86_64-linux-gnu/openblas-pthread/libopenblasp-r0.3.20.so;  LAPACK
version 3.10.0
##
## locale:
##  [1] LC_CTYPE=en_US.UTF-8       LC_NUMERIC=C
##  [3] LC_TIME=en_US.UTF-8        LC_COLLATE=en_US.UTF-8
##  [5] LC_MONETARY=en_US.UTF-8    LC_MESSAGES=en_US.UTF-8
##  [7] LC_PAPER=en_US.UTF-8       LC_NAME=C
##  [9] LC_ADDRESS=C               LC_TELEPHONE=C
## [11] LC_MEASUREMENT=en_US.UTF-8 LC_IDENTIFICATION=C
##
## time zone: Etc/UTC
## tzcode source: system (glibc)
##
## attached base packages:
## [1] stats4    stats     graphics  grDevices utils     datasets  methods
## [8] base
##
## other attached packages:
##  [1] scales_1.2.1                caret_6.0-94
##  [3] lattice_0.21-8              viridis_0.6.2
##  [5] viridisLite_0.4.1           scran_1.28.0
##  [7] BiocParallel_1.34.0         bluster_1.10.0
##  [9] batchelor_1.16.0            scater_1.28.0
## [11] scuttle_1.10.0              patchwork_1.1.2
## [13] dittoSeq_1.12.0             pheatmap_1.0.12
## [15] CATALYST_1.24.0             cytomapper_1.12.0
## [17] EBImage_4.42.0              lubridate_1.9.2
## [19] forcats_1.0.0               stringr_1.5.0
## [21] dplyr_1.1.2                 purrr_1.0.1
## [23] readr_2.1.4                 tidyr_1.3.0
## [25] tibble_3.2.1                ggplot2_3.4.2
## [27] tidyverse_2.0.0             imcRtools_1.6.0
## [29] SpatialExperiment_1.10.0    SingleCellExperiment_1.22.0
## [31] SummarizedExperiment_1.30.0 Biobase_2.60.0
## [33] GenomicRanges_1.52.0        GenomeInfoDb_1.36.0
## [35] IRanges_2.34.0              S4Vectors_0.38.0
## [37] BiocGenerics_0.46.0         MatrixGenerics_1.12.0
## [39] matrixStats_0.63.0          BiocStyle_2.28.0
##
## loaded via a namespace (and not attached):
```

```
##   [1] R.methodsS3_1.8.2          vroom_1.6.1
##   [3] tiff_0.1-11                nnet_7.3-18
##   [5] DT_0.27                    HDF5Array_1.28.0
##   [7] TH.data_1.1-2              vctrs_0.6.2
##   [9] digest_0.6.31              png_0.1-8
##  [11] shape_1.4.6                proxy_0.4-27
##  [13] ggrepel_0.9.3              parallelly_1.35.0
##  [15] magick_2.7.4               MASS_7.3-58.4
##  [17] reshape2_1.4.4             httpuv_1.6.9
##  [19] foreach_1.5.2              withr_2.5.0
##  [21] xfun_0.39                  ggpubr_0.6.0
##  [23] ellipsis_0.3.2             survival_3.5-5
##  [25] RTriangle_1.6-0.12         ggbeeswarm_0.7.1
##  [27] RProtoBufLib_2.12.0        drc_3.0-1
##  [29] systemfonts_1.0.4          ragg_1.2.5
##  [31] zoo_1.8-12                 GlobalOptions_0.1.2
##  [33] gtools_3.9.4               V8_4.3.0
##  [35] R.oo_1.25.0                promises_1.2.0.1
##  [37] rstatix_0.7.2              globals_0.16.2
##  [39] rhdf5filters_1.12.0        rhdf5_2.44.0
##  [41] rstudioapi_0.14            units_0.8-2
##  [43] generics_0.1.3             concaveman_1.1.0
##  [45] curl_5.0.0                 zlibbioc_1.46.0
##  [47] ScaledMatrix_1.7.1         ggraph_2.1.0
##  [49] polyclip_1.10-4            randomForest_4.7-1.1
##  [51] GenomeInfoDbData_1.2.10    fftwtools_0.9-11
##  [53] xtable_1.8-4               doParallel_1.0.17
##  [55] evaluate_0.20              hms_1.1.3
##  [57] bookdown_0.33              irlba_2.3.5.1
##  [59] colorspace_2.1-0           magrittr_2.0.3
##  [61] later_1.3.0                future.apply_1.10.0
##  [63] XML_3.99-0.14              cowplot_1.1.1
##  [65] RcppAnnoy_0.0.20           class_7.3-21
##  [67] svgPanZoom_0.3.4           pillar_1.9.0
##  [69] nlme_3.1-162               iterators_1.0.14
##  [71] compiler_4.3.0             beachmat_2.16.0
##  [73] stringi_1.7.12             gower_1.0.1
##  [75] sf_1.0-12                  plyr_1.8.8
##  [77] crayon_1.5.2               abind_1.4-5
##  [79] locfit_1.5-9.7             sp_1.6-0
##  [81] graphlayouts_0.8.4         bit_4.0.5
##  [83] terra_1.7-29               sandwich_3.0-2
##  [85] codetools_0.2-19           multcomp_1.4-23
##  [87] textshaping_0.3.6          recipes_1.0.6
##  [89] BiocSingular_1.16.0        bslib_0.4.2
##  [91] e1071_1.7-13               GetoptLong_1.0.5
##  [93] mime_0.12                  splines_4.3.0
##  [95] circlize_0.4.15            Rcpp_1.0.10
##  [97] sparseMatrixStats_1.12.0   knitr_1.42
##  [99] utf8_1.2.3                 clue_0.3-64
## [101] listenv_0.9.0              nnls_1.4
## [103] DelayedMatrixStats_1.22.0  ggsignif_0.6.4
## [105] Matrix_1.5-4               statmod_1.5.0
## [107] tzdb_0.3.0                 svglite_2.1.1
```

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## [109] tweenr_2.0.2               pkgconfig_2.0.3
## [111] tools_4.3.0                cachem_1.0.7
## [113] DBI_1.1.3                  fastmap_1.1.1
## [115] rmarkdown_2.21             grid_4.3.0
## [117] shinydashboard_0.7.2       broom_1.0.4
## [119] sass_0.4.5                 BiocManager_1.30.20
## [121] carData_3.0-5              rpart_4.1.19
## [123] farver_2.1.1               tidygraph_1.2.3
## [125] yaml_2.3.7                 cli_3.6.1
## [127] lifecycle_1.0.3            uwot_0.1.14
## [129] mvtnorm_1.1-3              lava_1.7.2.1
## [131] backports_1.4.1            DropletUtils_1.20.0
## [133] cytolib_2.12.0             timechange_0.2.0
## [135] gtable_0.3.3               rjson_0.2.21
## [137] ggridges_0.5.4             parallel_4.3.0
## [139] pROC_1.18.0                limma_3.56.0
## [141] jsonlite_1.8.4             edgeR_3.42.0
## [143] bitops_1.0-7               bit64_4.0.5
## [145] Rtsne_0.16                 FlowSOM_2.8.0
## [147] BiocNeighbors_1.18.0       flowCore_2.12.0
## [149] jquerylib_0.1.4            highr_0.10
## [151] metapod_1.8.0              dqrng_0.3.0
## [153] R.utils_2.12.2             timeDate_4022.108
## [155] shiny_1.7.4                ConsensusClusterPlus_1.64.0
## [157] htmltools_0.5.5            distances_0.1.9
## [159] glue_1.6.2                 ResidualMatrix_1.10.0
## [161] XVector_0.40.0             RCurl_1.98-1.12
## [163] classInt_0.4-9             jpeg_0.1-10
## [165] gridExtra_2.3              igraph_1.4.2
## [167] R6_2.5.1                   labeling_0.4.2
## [169] cluster_2.1.4              Rhdf5lib_1.22.0
## [171] ipred_0.9-14               DelayedArray_0.25.0
## [173] tidyselect_1.2.0           vipor_0.4.5
## [175] plotrix_3.8-2              ggforce_0.4.1
## [177] raster_3.6-20              car_3.1-2
## [179] future_1.32.0              ModelMetrics_1.2.2.2
## [181] rsvd_1.0.5                 munsell_0.5.0
## [183] KernSmooth_2.23-20         data.table_1.14.8
## [185] htmlwidgets_1.6.2          ComplexHeatmap_2.16.0
## [187] RColorBrewer_1.1-3         rlang_1.1.0
## [189] colorRamps_2.3.1           ggnewscale_0.4.8
## [191] fansi_1.0.4                hardhat_1.3.0
## [193] beeswarm_0.4.0             prodlim_2023.03.31
```

Supplementary Note 2

Spillover slide preparation:

- Prepare 2% agarose in double distilled H_2O in a beaker and melt it in a microwave until well dissolved.
- Dip a blank superfrost plus glass microscope slide into the agarose and submerge it until the label.
- Remove the slide and prop it up against a support to allow the excess agarose to run off onto paper towels.
- Allow the slide to dry completely (at least 30 minutes).
- Retrieve all the antibody conjugates used in the panel for which the spillover matrix is to be generated and place them on ice.
- Arrange them in a known order (e.g. mass of the conjugated metal).
- Pipette 0.3 µl spots of 0.4% trypan blue dye into an array on the slide. Prepare one spot per antibody, and make sure the spots are well separated.
- Pipette 0.3 µl of each antibody conjugate (usually at 0.5 mg/ml) onto a unique blue spot, taking care to avoid different antibodies bleeding into each other. Note the exact location of each conjugate on the slide.
- Let the spots dry completely, at least 1 hour.

Spillover slide acquisition:

- Create a JPEG or PNG image of the slide using a mobile phone camera or flat-bed scanner.
- In the CyTOF software, create a new file and import the slide image into it.
- Create a panorama across all the spots to visualize their locations.
- Within each spot, create a region of interest (ROI) with a width of 200 pixels and a height of 10 pixels.
- Name each ROI with the mass and name of the metal conjugate contained in the spot, e.g., “Ir193” or “Ho165”. This will be how each TXT file is named.
- Set the profiling type of each ROI to “Local”.
- Apply the antibody panel to all the ROIs. This panel should contain all (or more) of the isotopes in the panel, with the correct metal specified. For example: if the metal used is Barium 138, make sure this, rather than Lanthanum 138, is selected.
- Save the file, make sure “Generate Text File” is selected, and start the acquisition.