Seurat pipeline 8 um data analysis

Code

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2025-05-06

Seurat pipeline

Following Seurat “Analysis, visualization, and integration of Visium HD spatial datasets with Seurat” tutorial, available in: https://satijalab.org/seurat/articles/visiumhd_analysis_vignette (https://satijalab.org/seurat/articles/visiumhd_analysis_vignette).

And the “Visium HD Analysis” from Harvard Chan Bioinformatics Core (HBC) (http://bioinformatics.sph.harvard.edu/). Available in: https://github.com/hbctraining/spatial_nanocourse/blob/main/lessons/visium_hd.md (https://github.com/hbctraining/spatial_nanocourse/blob/main/lessons/visium_hd.md)

Data used available in the 10X Genomics Data Base in: https://www.10xgenomics.com/datasets/visium-hd-cytassist-gene-expression-human-breast-cancer-fresh-frozen (https://www.10xgenomics.com/datasets/visium-hd-cytassist-gene-expression-human-breast-cancer-fresh-frozen)

Hide

```
# Necessary packages CRAN
list.of.packages = c("Seurat","ggplot2", "dplyr", "patchwork","hdf5r","arrow","ape", "remotes", "devtools", "tictoc")

# Install necessary packages if needed
new.packages = list.of.packages[!(list.of.packages %in% installed.packages())]
if(length(new.packages)> 0) install.packages(new.packages)

# Load CRAN packages
invisible(lapply(list.of.packages, FUN=library, character.only=TRUE))
```

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```
# Verify if Seurat packages are installed
if (!requireNamespace("SeuratDisk", quietly = TRUE)) {
  remotes::install_github("mojaveazure/seurat-disk")
}
```

Hide

```
if (!requireNamespace("SeuratData", quietly = TRUE)) {
  remotes::install_github("satijalab/seurat-data")
}

# Load Seurat packages
invisible(lapply(c("SeuratDisk", "SeuratData"), FUN=library, character.only=TRUE))

# Bioconductor packages
bioconductor.packages = c("GO.db", "org.Hs.eg.db", "limma")

if (!requireNamespace("BiocManager", quietly = TRUE)) {
  install.packages("BiocManager", repos = "https://cloud.r-project.org")
}
library(BiocManager)
```

Hide

```
new.packages.bio = bioconductor.packages[!(bioconductor.packages %in% installed.packages())]
if(length(new.packages.bio)> 0) BiocManager::install(new.packages.bio)

# Load Bioconductor packages
invisible(lapply(bioconductor.packages, FUN=library, character.only=TRUE))
```

Hide

```
# Measure run time notebook
tic("Total time Seurat pipeline")
```

Hide

```
# Load Visium HD data
localdir <- "../../Data/Raw/raw_data_fresh_frozen/"
list.files(localdir)
```

```
[1] "binned_outputs"                                          "spatial"
[3] "Visium_HD_Human_Breast_Cancer_Fresh_Frozen_tissue_image.tif"
```

Hide

```
# Load a 10x Genomics 8 um Visium Spatial Experiment into a Seurat object
object <- Load10X_Spatial(data.dir = localdir, bin.size = 8)
object
```

```
An object of class Seurat
18085 features across 472859 samples within 1 assay
Active assay: Spatial.008um (18085 features, 0 variable features)
 1 layer present: counts
 1 spatial field of view present: slice1.008um
```

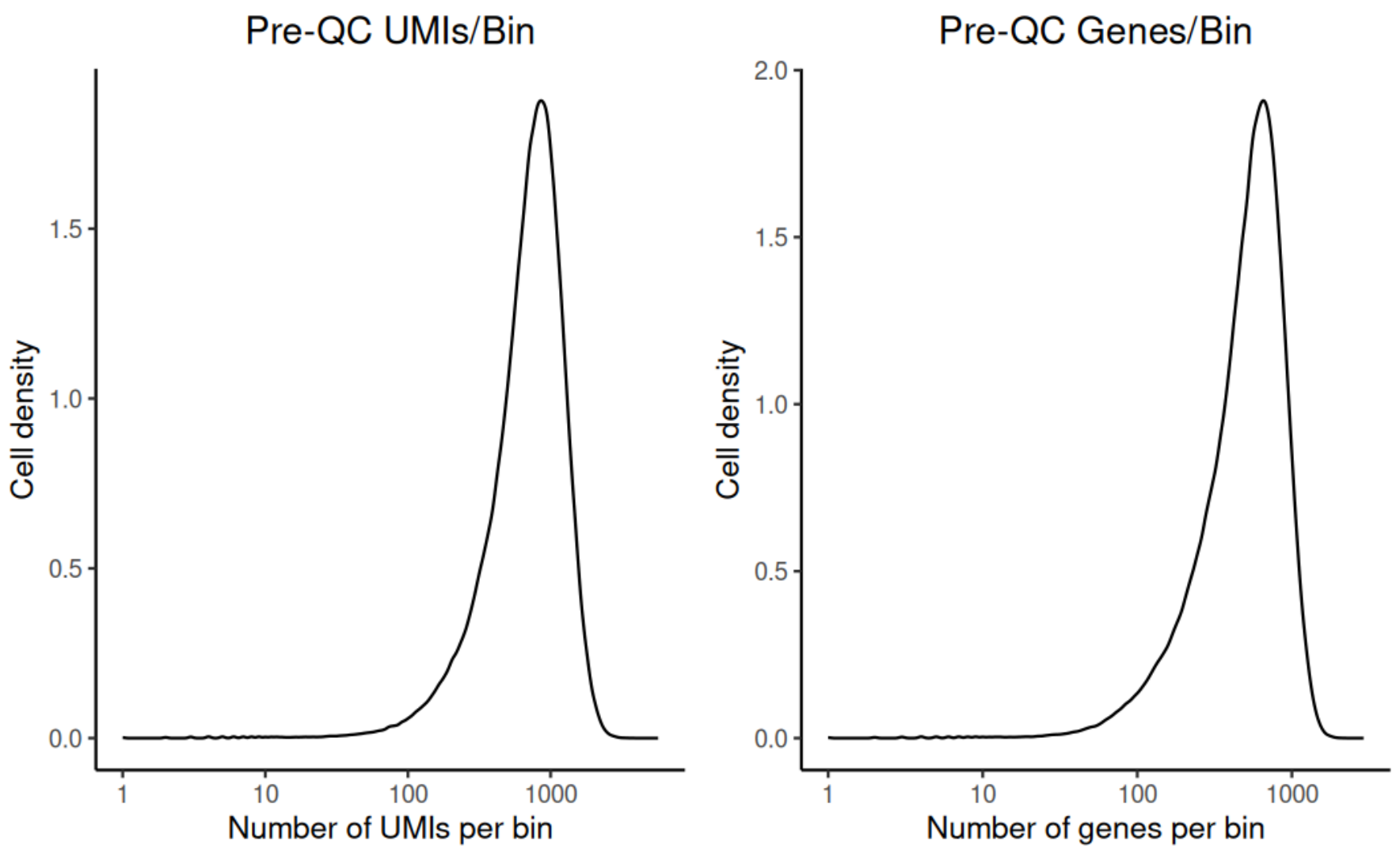
Hide

```
# Quality Control
# Pre-filterning
# Create a metadata object
object_meta <- object@meta.data

# Plot the number of UMIs (nUMI) and the number of genes (nGene)
# Create a plot for nUMI
dist_counts_before <- object_meta %>%
  ggplot(aes(x=nCount_Spatial.008um)) +
  geom_density(alpha = 0.2) +
  scale_x_log10() +
  theme_classic() +
  ylab("Cell density") +
  xlab("Number of UMIs per bin") +
  ggtitle('Pre-QC UMIs/Bin') +
  theme(plot.title = element_text(hjust = 0.5))

# Create a plot for nGene
dist_features_before <- object_meta %>%
  ggplot(aes(x=nFeature_Spatial.008um)) +
  geom_density(alpha = 0.2) +
  scale_x_log10() +
  theme_classic() +
  ylab("Cell density") +
  xlab("Number of genes per bin") +
  ggtitle('Pre-QC Genes/Bin') +
  theme(plot.title = element_text(hjust = 0.5))

dists_before <- dist_counts_before | dist_features_before
dists_before
```



Hide

```
# Good quality data, just one pick representing healthy cells with high number of genes and UMIs per bin.
```

Hide

```
# Apply filter to delete low quality bins and genes
print(paste("The numer of initial bins and genes before filtering was:",  nrow(object@meta.data), "and",nrow(obje
ct), "respectively." ))
```

```
[1] "The numer of initial bins and genes before filtering was: 472859 and 18085 respectively."
```

Hide

```
# Create a filtered object, with with nUMI > 80 and nGene > 80, leaving the higher quality bins
object_filt <- subset(object, (nCount_Spatial.008um > 80) &
                        (nFeature_Spatial.008um > 80))


# Calculate the % of mitocondrial genes per bin
object_filt[["percent.mt"]] <- PercentageFeatureSet(object_filt, pattern = "^MT-")

# Apply filter keeping bins of < 30% mitocondrial genes
object_filt <- subset(object_filt, subset = percent.mt < 30)

# Obtain the count matrix
counts = GetAssayData(object_filt,layer = "counts")
# Filter genes that appear in at least 5 bins, high quality genes
hq_genes = rowSums(counts >0) >= 5
# Subset seurat object to keep only high quality genes
object_filt = subset(object_filt, features = names(hq_genes[hq_genes]))
print(paste("Resulting in" , nrow(object_filt@meta.data), "bins and",nrow(object_filt) , "genes after filtering f
or further processing."))
```

```
[1] "Resulting in 463329 bins and 16375 genes after filtering for further processing."
```

Hide

```
# Calculate statistics of filtered object
summary(object_filt$nCount_Spatial.008um, na.rm = T)
```

```
   Min. 1st Qu.  Median    Mean 3rd Qu.    Max. 
     82     506     747     787    1015    5670 
```

Hide

```
summary(object_filt$nFeature_Spatial.008um, na.rm = T)
```

```
   Min. 1st Qu.  Median    Mean 3rd Qu.    Max. 
   81.0   355.0   544.0   560.5   735.0  2913.0 
```

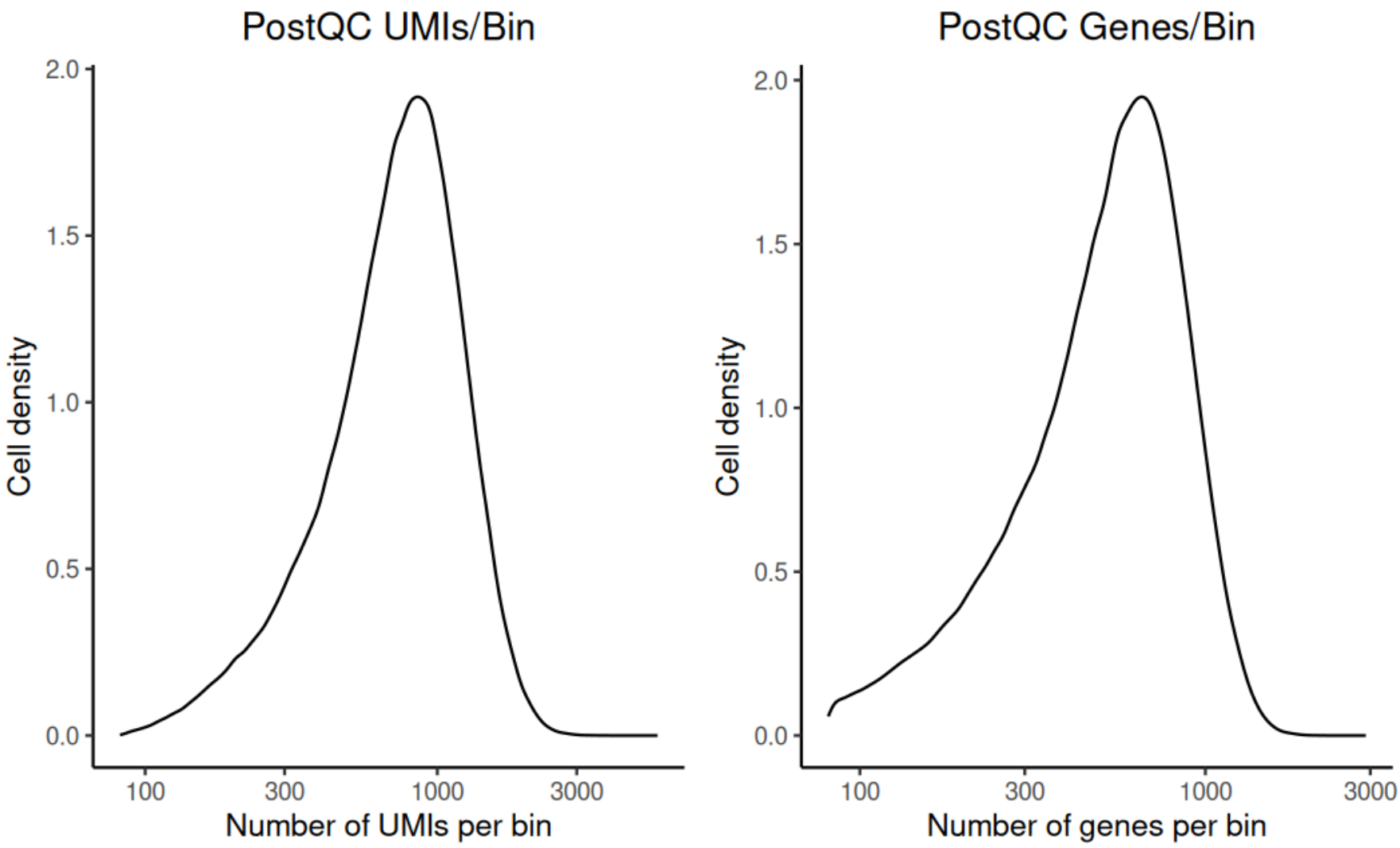
Hide

```
# Create a new metadata data frame with the filtered object
object_filt_meta <- object_filt@meta.data

# Plot nUMI
dist_counts_after <- object_filt_meta %>%
  ggplot(aes(x=nCount_Spatial.008um)) +
  geom_density(alpha = 0.2) +
  scale_x_log10() +
  theme_classic() +
  ylab("Cell density") +
  xlab("Number of UMIs per bin") +
  ggtitle('PostQC UMIs/Bin') +
  theme(plot.title = element_text(hjust = 0.5))

# Plot nGene
dist_features_after <- object_filt_meta %>%
  ggplot(aes(x=nFeature_Spatial.008um)) +
  geom_density(alpha = 0.2) +
  scale_x_log10() +
  theme_classic() +
  ylab("Cell density") +
  xlab("Number of genes per bin") +
  ggtitle('PostQC Genes/Bin') +
  theme(plot.title = element_text(hjust = 0.5))

# Combine plots side-by-side
dists_after <- dist_counts_after | dist_features_after
dists_after
```



Hide

```
# Visualizing Counts Data
# Visualize the number of UMIs and gene counts per bin as a distribution
# Violin plot of UMI counts
vln_counts_after <- VlnPlot(object_filt,
                            features = "nCount_Spatial.008um",
                            pt.size = 0,
                            group.by = 'orig.ident') +
  NoLegend()  + scale_y_log10() + ggtitle('nUMI') + xlab('FF_breast') + ylim(c(80, 6000))
```

```
Warning: Default search for "data" layer in "Spatial.008um" assay yielded no results; utilizing "counts" layer in
stead.Warning: The `slot` argument of `FetchData()` is deprecated as of SeuratObject 5.0.0.
Please use the `layer` argument instead.Warning: `PackageCheck()` was deprecated in SeuratObject 5.0.0.
Please use `rlang::check_installed()` instead.Scale for y is already present.
Adding another scale for y, which will replace the existing scale.Scale for y is already present.
Adding another scale for y, which will replace the existing scale.
```

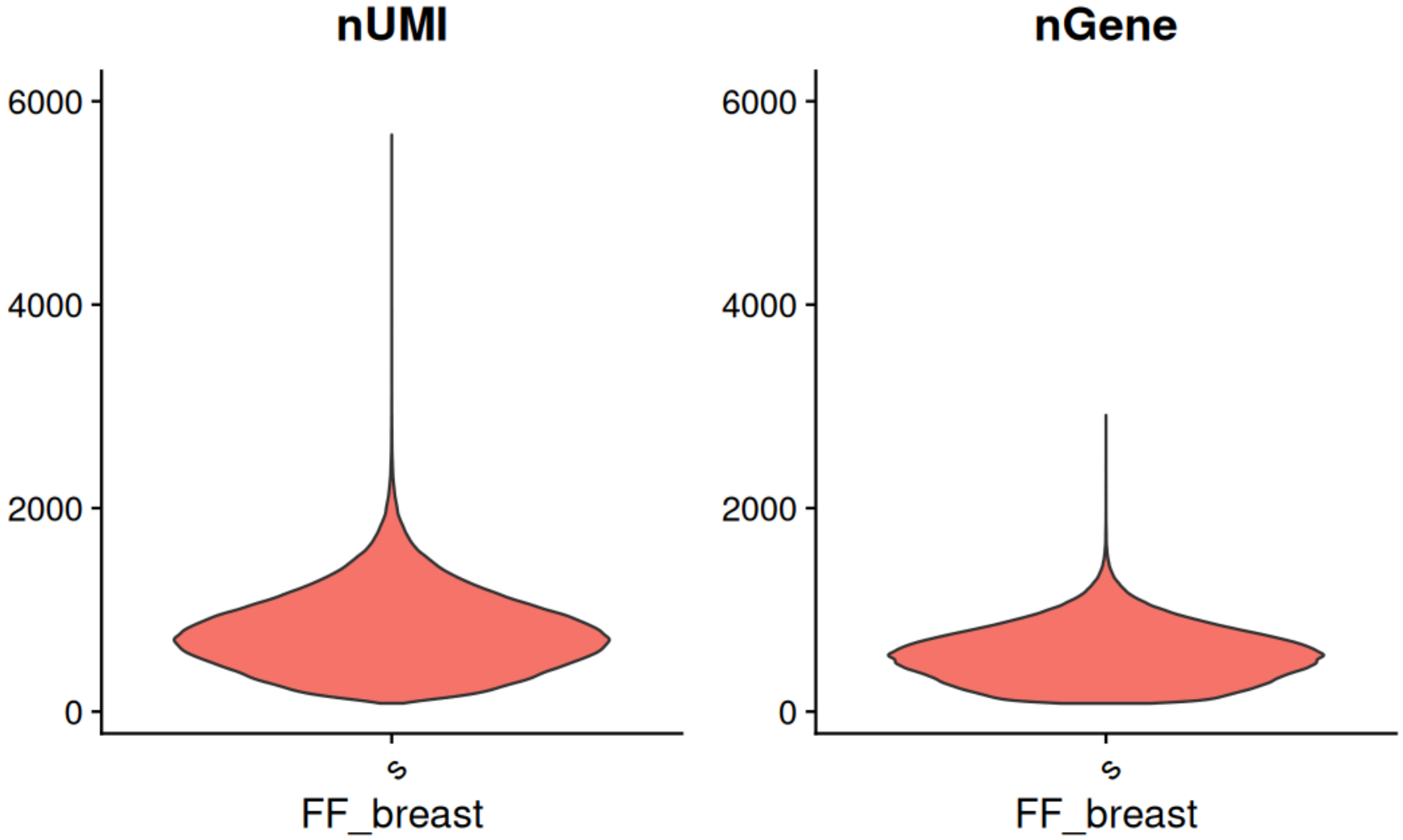
Hide

```
# Violin plot of gene counts
vln_features_after <- VlnPlot(object_filt,
                            features = "nFeature_Spatial.008um",
                            pt.size = 0,
                            group.by = 'orig.ident') +
  NoLegend() + scale_y_log10() + ggtitle('nGene') +  xlab('FF_breast') + ylim(c(80, 6000))
```

```
Warning: Default search for "data" layer in "Spatial.008um" assay yielded no results; utilizing "counts" layer in
stead.Scale for y is already present.
Adding another scale for y, which will replace the existing scale.Scale for y is already present.
Adding another scale for y, which will replace the existing scale.
```

Hide

```
# Plot both side by side
vln_counts_after | vln_features_after
```



Hide

```
# Visualizing UMI count across the tissue image
image_counts <- SpatialFeaturePlot(object_filt,
                                   feature = 'nCount_Spatial.008um',
                                   pt.size.factor = 8) +
  theme(legend.position = "top",
        legend.title = element_text(size = 10, hjust = 0, vjust = 1),
        legend.text = element_text(size = 6.5))


# Visualizing gene count across the image
image_features <- SpatialFeaturePlot(object_filt,
                                     features = "nFeature_Spatial.008um",
                                     pt.size.factor = 8) +
  theme(legend.position = "top",
        legend.title = element_text(size = 10, hjust = 0, vjust = 1))


# Plot the two side-by-side
image_counts | image_features
```

Hide

```
# note that many spots have very few counts, in-part
# due to low cellular density in certain tissue regions
```

Hide

```
# Normalize dataset, use standard log-normalization for spatial data
object_filt <- NormalizeData(object_filt, assay = 'Spatial.008um')
```

```
Normalizing layer: counts
Performing log-normalization
0%   10   20   30   40   50   60   70   80   90   100%
[----|----|----|----|----|----|----|----|----|----|
**************************************************|
```

Hide

```
object_filt
```

```
An object of class Seurat
16375 features across 463329 samples within 1 assay
Active assay: Spatial.008um (16375 features, 0 variable features)
 2 layers present: counts, data
 1 spatial field of view present: slice1.008um
```

Hide

```
# Unsupervised clustering
# Define a set of highly variable genes, will help to quantify the variability and similarity between bins.
object_filt <- FindVariableFeatures(object_filt)
```

```
Finding variable features for layer counts
Calculating gene variances
0%   10   20   30   40   50   60   70   80   90   100%
[----|----|----|----|----|----|----|----|----|----|
**************************************************|
Calculating feature variances of standardized and clipped values
0%   10   20   30   40   50   60   70   80   90   100%
[----|----|----|----|----|----|----|----|----|----|
**************************************************|
```

Hide

```
object_filt <- ScaleData(object_filt)
```

```
Centering and scaling data matrix

  |
  |
|   0%
  |
  |=======================================================================================
|  50%
  |
  |=======================================================================================
=============================================================| 100%
```

Hide

```
# Select 50,0000 cells and create a new 'sketch' assay
object_filt <- SketchData(
  object = object_filt,
  ncells = 50000,
  method = "LeverageScore",
  sketched.assay = "sketch",
  features = VariableFeatures(object_filt)
)
```

```
Calcuating Leverage Score
Attempting to cast layer counts to dgCMatrix
Attempting to cast layer data to dgCMatrix
```

Hide

```
object_filt
```

```
An object of class Seurat
32750 features across 463329 samples within 2 assays
Active assay: sketch (16375 features, 2000 variable features)
 2 layers present: counts, data
 1 other assay present: Spatial.008um
 1 spatial field of view present: slice1.008um
```

Hide

```
# Observe the leverage score has been added as a column to the metadata of our object.
head(object_filt@meta.data)
```

	orig.ident <chr>	nCount_Spatial.008um <dbl>	nFeature_Spatial.008um <int>	percent.mt <dbl>
s_008um_00269_00526-1	s	1479	1023	5.814740
s_008um_00260_00253-1	s	971	719	7.106076
s_008um_00433_00599-1	s	527	214	1.328273
s_008um_00266_00304-1	s	749	547	6.275033
s_008um_00359_00037-1	s	999	721	8.608609
s_008um_00469_00254-1	s	606	470	10.231023

6 rows | 1-5 of 5 columns

Hide

```
# Perform clustering workflow
object_filt <- FindVariableFeatures(object_filt)
```

```
Finding variable features for layer counts
Calculating gene variances
0%   10   20   30   40   50   60   70   80   90   100%
[----|----|----|----|----|----|----|----|----|----|
**************************************************|
Calculating feature variances of standardized and clipped values
0%   10   20   30   40   50   60   70   80   90   100%
[----|----|----|----|----|----|----|----|----|----|
**************************************************|
```

Hide

```
object_filt <- ScaleData(object_filt)
```

```
Centering and scaling data matrix

  |
  |
|   0%
  |
  |=======================================================================================
|  50%
  |
  |=======================================================================================
=============================================================| 100%
```

Hide

```
object_filt <- RunPCA(object_filt, assay = "sketch", reduction.name = "pca.sketch")
```

```
PC_ 1
Positive:  APOD, PIP, CLU, TSKU, ABCC11, PNMT, LTF, SULT1C3, MAB21L4, MPV17L
       S100A9, TAT, FABP7, PPP1R1B, UGT2B28, ANKRD30A, SCD, AQP3, ELF3, KRT8
       ZNF652, ARFGEF3, S100A7A, AR, TACSTD2, CLDN4, PEG10, ABCA12, CLDN3, IRX3
Negative:  SPARC, COL1A1, COL3A1, COL1A2, IGKC, IGHG1, IGFBP7, COL4A1, COL6A2, FN1
       COL4A2, BGN, A2M, TIMP1, LUM, COL6A1, TAGLN, AEBP1, CALD1, APOE
       COL18A1, MMP2, COL6A3, ACTA2, DCN, PRSS23, THY1, COL5A2, COL5A1, PLVAP
PC_ 2
Positive:  LYZ, APOE, IGKC, IGHG1, C1QC, C1QA, CD68, FTL, C3, C1QB
       CTSB, CTSZ, CTSS, GPNMB, LAPTM5, CTSD, IGHA1, TYROBP, LUM, SPI1
       MZB1, LSP1, MS4A6A, DCN, CYBB, MPEG1, CD4, LCP1, TRAC, IGHG3
Negative:  PLVAP, MCAM, COL18A1, COL4A1, AQP1, COL4A2, VWF, CD34, ENG, PODXL
       RGS5, EGFL7, IGFBP7, CALCRL, CDH5, ESM1, PLPP1, ESAM, SLC9A3R2, KDR
       NOTCH3, CD93, RAMP2, SPARCL1, SEMA3F, EPAS1, OLFML2A, DLL4, A2M, EXOC3L2
PC_ 3
Positive:  IGKC, IGHG1, IGHA1, IGLC1, IGHG3, MZB1, DERL3, IGHM, IGHD, TENT5C
       PIM2, TXNDC5, JCHAIN, POU2AF1, CD79A, FCRL5, ITM2C, SSR4, TNFRSF17, SEL1L3
       TXNDC11, CD27, DPEP1, LAX1, BMP6, BTG2, P2RX1, F13A1, CCR2, CPA3
Negative:  CTSB, LYZ, APOE, CTSD, CXCL10, CTSZ, CXCL9, CD68, APOC1, LAPTM5
       LGMN, CXCL11, CTSL, GRN, LCP1, GPNMB, ACP5, S100A9, CTSS, FTL
       GBP1, IL4I1, TYROBP, LHFPL2, C3, SPI1, CLU, LIPA, TOP2A, SLC15A3
PC_ 4
Positive:  LYZ, CD68, LAPTM5, LCP1, APOE, ENG, ITGAX, PLVAP, VWF, PLEK
       A2M, CTSZ, CTSS, CTSD, CALCRL, CD34, PECAM1, LSP1, EGFL7, SPI1
       CD4, RAMP2, CYBB, TNFAIP2, C1QC, CD83, IL4I1, MPEG1, CDH5, TRAC
Negative:  AEBP1, COL1A2, CCN2, COL12A1, COMP, COL1A1, LUM, COL5A1, COL11A1, THBS2
       COL6A3, COL3A1, COL5A2, FN1, SFRP2, THBS1, FBN1, DCN, LRRC15, CTHRC1
       VCAN, POSTN, COL8A1, SULF1, C1S, EPYC, MXRA5, MMP11, BGN, C1R
PC_ 5
Positive:  TOP2A, CDK1, TPX2, NUSAP1, ASPM, ANLN, HIST1H1B, HMGB2, MYBL2, MKI67
       KIFC1, TROAP, CIT, FAM83D, HIST1H1D, SPC24, ECT2, CENPF, CCNB1, IGKC
       FOXM1, UBE2C, CCNA2, CCNB2, IGHG1, CDCA3, PLK1, PRC1, SPAG5, GPSM2
Negative:  SULT1C3, SLC26A3, APOD, TAT, TSKU, PIP, ACSM1, ECHDC2, MPV17L, FABP7
       ACSL3, ATP13A4, CYP1B1, ZBTB16, SLPI, ABCC11, AQP3, UGT2B28, GPCPD1, KYNU
       HMGCS2, THRSP, SORD, FMO5, ZNF652, UGT2B11, MYCBP2, ABCA12, IRX3, SOD2
```

Hide

```
object_filt <- FindNeighbors(object_filt, assay = "sketch", reduction = "pca.sketch", dims = 1:50)
```

```
Computing nearest neighbor graph
Computing SNN
```

Hide

```
# Find CLusters with Leiden algorith
object_filt <- FindClusters(object_filt, cluster.name = "seurat_cluster.sketched", resolution = 0.65, algorithm =
4)
```

```
Warning: `random.seed` must be greater than 0 for leiden clustering, resetting `random.seed` to 1.3 singletons id
entified. 12 final clusters.
```

Hide

```
# Create a UMAP using the principal components as input
object_filt <- RunUMAP(object_filt, reduction = "pca.sketch", reduction.name = "umap.sketch", return.model = T, d
ims = 1:50)
```

```
Warning: The default method for RunUMAP has changed from calling Python UMAP via reticulate to the R-native UWOT
using the cosine metric
To use Python UMAP via reticulate, set umap.method to 'umap-learn' and metric to 'correlation'
This message will be shown once per sessionUMAP will return its model
20:41:53 UMAP embedding parameters a = 0.9922 b = 1.112
20:41:53 Read 50000 rows and found 50 numeric columns
20:41:53 Using Annoy for neighbor search, n_neighbors = 30
20:41:53 Building Annoy index with metric = cosine, n_trees = 50
0%   10   20   30   40   50   60   70   80   90   100%
[----|----|----|----|----|----|----|----|----|----|
**************************************************|
20:41:59 Writing NN index file to temp file /tmp/Rtmpyu4MmB/file29a6c692c2a
20:41:59 Searching Annoy index using 1 thread, search_k = 3000
20:42:16 Annoy recall = 100%
20:42:17 Commencing smooth kNN distance calibration using 1 thread with target n_neighbors = 30
20:42:19 Initializing from normalized Laplacian + noise (using RSpectra)
20:42:21 Commencing optimization for 200 epochs, with 2452432 positive edges
20:42:21 Using rng type: pcg
Using method 'umap'
0%   10   20   30   40   50   60   70   80   90   100%
[----|----|----|----|----|----|----|----|----|----|
**************************************************|
20:42:35 Optimization finished
```

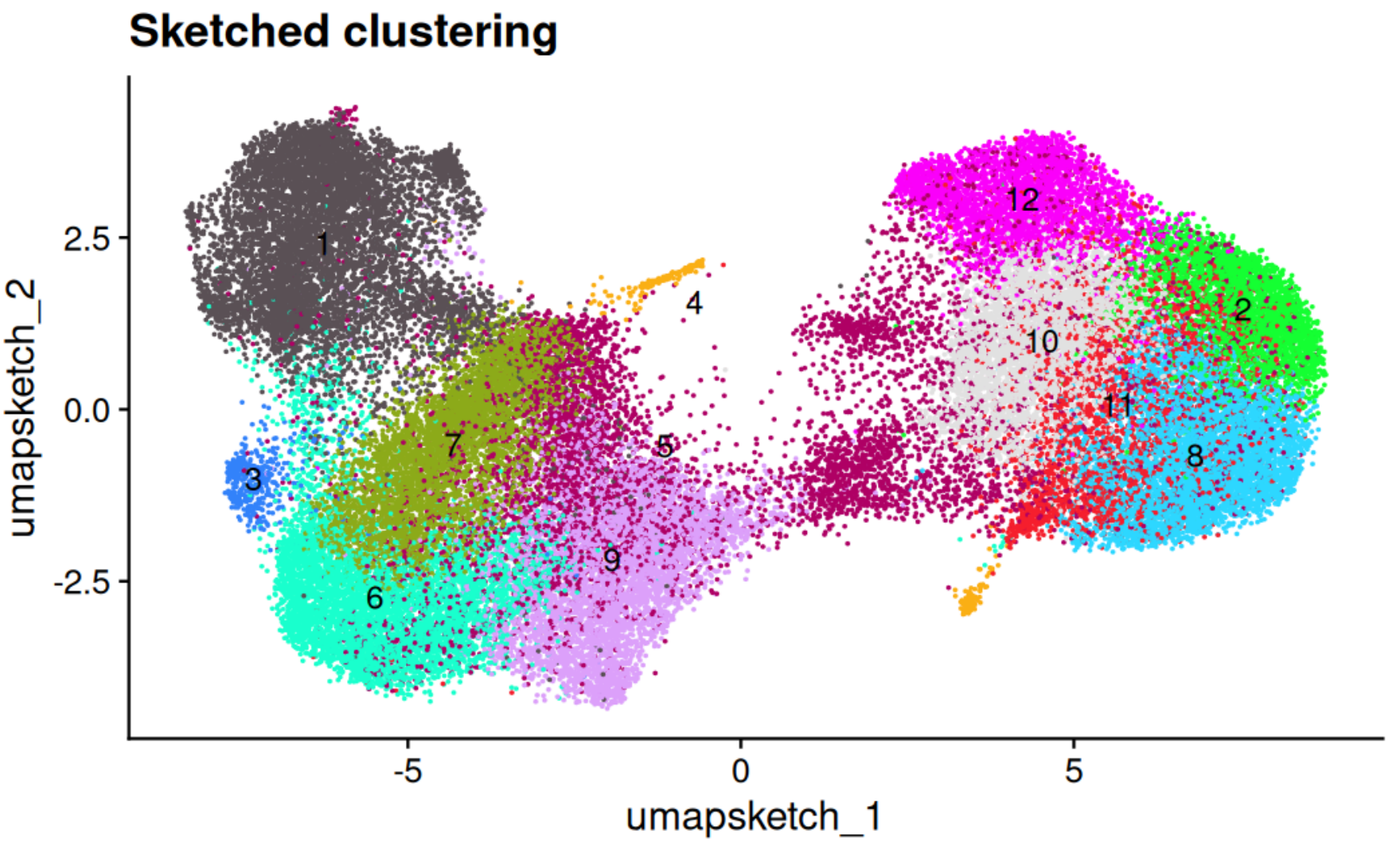
Hide

```
# Set color palette
color_pal <- Seurat::DiscretePalette(n = length(unique(object_filt$seurat_cluster.sketched)),
                                     palette = "polychrome")
names(color_pal) <- sort(unique(object_filt$seurat_cluster.sketched))

# Arrange so clusters get listed in numerical order
object_filt$seurat_cluster.sketched <- object_filt$seurat_cluster.sketched %>%
  as.numeric %>% as.factor()

Idents(object_filt) <- "seurat_cluster.sketched"

# Plot UMAP
DimPlot(object_filt, reduction = "umap.sketch", label = T, cols = color_pal) +
  ggtitle("Sketched clustering") +
  theme(legend.position = "none")
```



Hide

```
# Adjust MaxSize to run next step
options(future.globals.maxSize= 2000000000)

# Project the cluster labels, and dimensional reductions (PCA and UMAP) that we learned from the 50,000 sketched
cells
object_filt <- ProjectData(
  object = object_filt,
  assay = "Spatial.008um",
  full.reduction = "full.pca.sketch",
  sketched.assay = "sketch",
  sketched.reduction = "pca.sketch",
  umap.model = "umap.sketch",
  dims = 1:50,
  refdata = list(seurat_cluster.projected = "seurat_cluster.sketched")
)
```

```
full.pca.sketch is not in the object. Data from all cells will be projected to pca.sketch
Projecting cell embeddings
Finding sketch neighbors
Finding sketch weight matrix
0%   10   20   30   40   50   60   70   80   90   100%
[----|----|----|----|----|----|----|----|----|----|
**************************************************|
Transfering refdata from sketch
Projection to sketch umap
Running UMAP projection
20:46:09 Read 463329 rows
20:46:09 Processing block 1 of 1
20:46:09 Commencing smooth kNN distance calibration using 1 thread with target n_neighbors = 30
20:46:12 Initializing by weighted average of neighbor coordinates using 1 thread
20:46:14 Commencing optimization for 67 epochs, with 13899870 positive edges
Using method 'umap'
0%   10   20   30   40   50   60   70   80   90   100%
[----|----|----|----|----|----|----|----|----|----|
**************************************************|
20:46:39 Finished
Warning: Keys should be one or more alphanumeric characters followed by an underscore, setting key from full.uma
p.sketch to fullumapsketch_
```

Hide

```
# Arrange so clusters get listed in numerical order
object_filt$seurat_cluster.projected <- object_filt$seurat_cluster.projected %>%
  as.numeric %>% as.factor()

object_filt
```

```
An object of class Seurat
32750 features across 463329 samples within 2 assays
Active assay: sketch (16375 features, 2000 variable features)
 3 layers present: counts, data, scale.data
 1 other assay present: Spatial.008um
 4 dimensional reductions calculated: pca.sketch, umap.sketch, full.pca.sketch, full.umap.sketch
 1 spatial field of view present: slice1.008um
```

Hide

```
head(object_filt@meta.data)
```

	orig.ident <chr>	nCount_Spatial.008um <dbl>	nFeature_Spatial.008um <int>	percent.mt <dbl>
s_008um_00269_00526-1	s	1479	1023	5.814740
s_008um_00260_00253-1	s	971	719	7.106076
s_008um_00433_00599-1	s	527	214	1.328273
s_008um_00266_00304-1	s	749	547	6.275033
s_008um_00359_00037-1	s	999	721	8.608609
s_008um_00469_00254-1	s	606	470	10.231023

6 rows | 1-5 of 9 columns

Hide

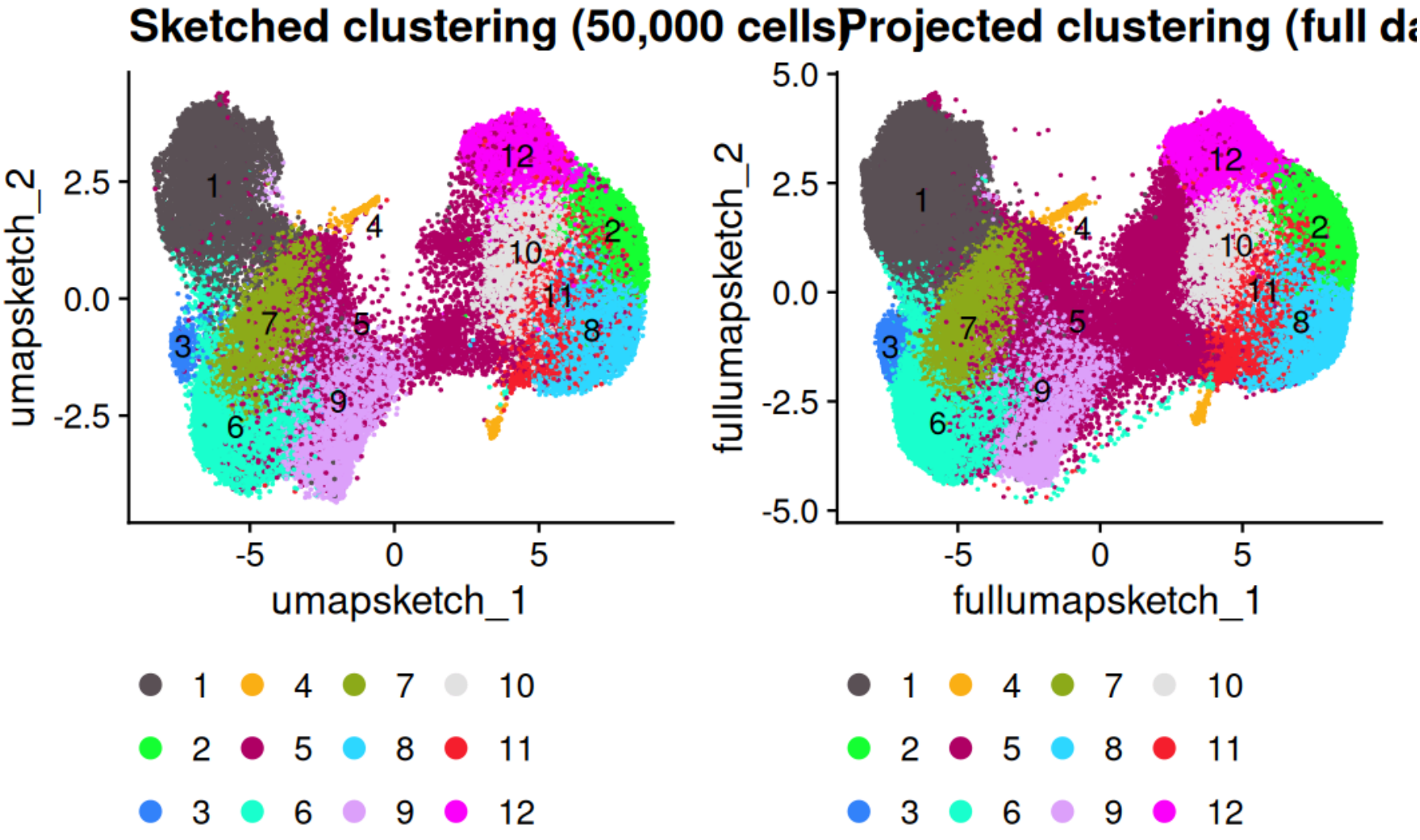
```
# Visualize the clustering results for the sketched cells, as well as the projected clustering results for the fu
ll dataset
DefaultAssay(object_filt) <- "sketch"
Idents(object_filt) <- "seurat_cluster.sketched"
p1 <- DimPlot(object_filt, reduction = "umap.sketch", label = T, raster = F, cols = color_pal) + ggtitle("Sketche
d clustering (50,000 cells)") + theme(legend.position = "bottom")

# switch to full dataset
DefaultAssay(object_filt) <- "Spatial.008um"
Idents(object_filt) <- "seurat_cluster.projected"
p2 <- DimPlot(object_filt, reduction = "full.umap.sketch", label = T, raster = F, cols = color_pal) + ggtitle("Pr
ojected clustering (full dataset)") + theme(legend.position = "bottom")

p_combinado <- p1 | p2
p_combinado
ggsave("p1.png", plot = p1, width = 8, height = 8, dpi = 300)
```

Hide

```
ggsave("p2.png", plot = p2, width = 8, height = 8, dpi = 300)
ggsave("p_combinado.png", plot = p_combinado, width = 12, height = 6, dpi = 300)
```

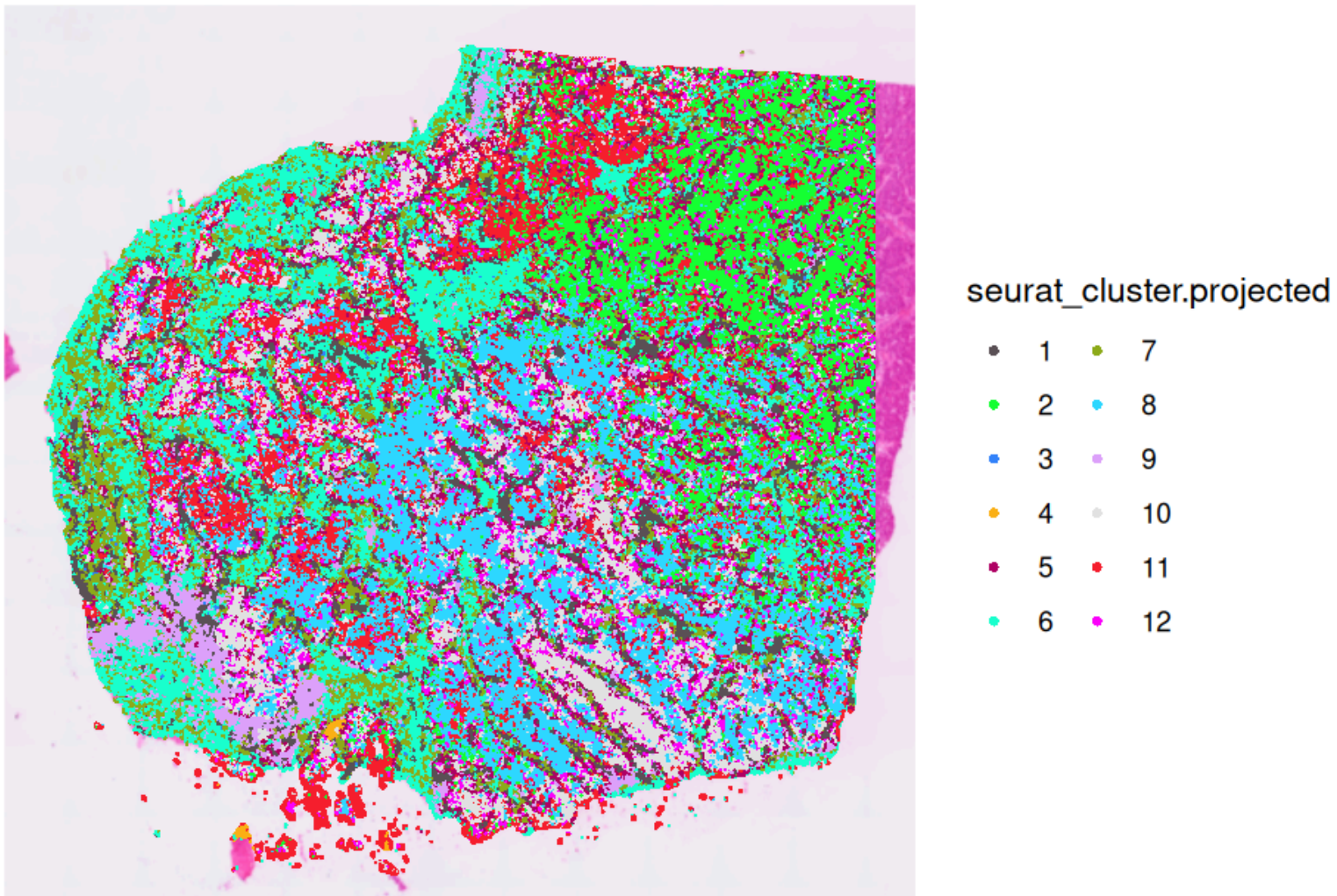


Hide

```
#  Visualize the unsupervised clusters based on their spatial location.
image_seurat_clusters <- SpatialDimPlot(object_filt,
                                        group.by = 'seurat_cluster.projected',
                                        pt.size.factor = 6, cols = color_pal) +
  guides(fill=guide_legend(ncol=2))

image_seurat_clusters

ggsave("clusters_image_0.65.png", plot = image_seurat_clusters, width = 8, height = 6, dpi = 300)
```



Hide

```
# Find and visualize the top gene expression markers for each cluster
# Crete downsampled object to make visualization easier
Idents(object_filt) <- "seurat_cluster.projected"
object_subset <- subset(object_filt, cells = Cells(object_filt[["Spatial.008um"]]), downsample = 1000)
```

```
Warning: Not validating Centroids objectsWarning: Not validating Centroids objectsWarning: Not validating FOV obj
ectsWarning: Not validating FOV objectsWarning: Not validating FOV objectsWarning: Not validating FOV objectsWarn
ing: Not validating FOV objectsWarning: Not validating FOV objectsWarning: Not validating Seurat objects
```

Hide

```
# Order clusters by similarity
DefaultAssay(object_subset) <- "Spatial.008um"
Idents(object_subset) <- "seurat_cluster.projected"
object_subset <- BuildClusterTree(object_subset, assay = "Spatial.008um", reduction = "full.pca.sketch", reorder
= T)
```

```
Reordering identity classes and rebuilding tree
```

Hide

```
markers <- FindAllMarkers(object_subset, assay = "Spatial.008um", only.pos = TRUE)
```

```
Calculating cluster 1
Warning: The `slot` argument of `GetAssayData()` is deprecated as of SeuratObject 5.0.0.
Please use the `layer` argument instead.For a (much!) faster implementation of the Wilcoxon Rank Sum Test,
(default method for FindMarkers) please install the presto package
--------------------------------------------
install.packages('devtools')
devtools::install_github('immunogenomics/presto')
--------------------------------------------
After installation of presto, Seurat will automatically use the more
efficient implementation (no further action necessary).
This message will be shown once per session
```

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|+++                                               | 6 % ~40s
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|+++++                                             | 9 % ~39s
|+++++                                             | 10% ~38s
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```

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```
Calculating cluster 9
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|                                                  | 0 % ~calculating
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```
Calculating cluster 5
```

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|                                                  | 0 % ~calculating
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  |++++++++++++++++++++++++++++++++++++++++          | 96% ~02s
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  |++++++++++++++++++++++++++++++++++++++++          | 100% elapsed=49s
```

```
Calculating cluster 4
```

```
|                                                  | 0 % ~calculating
|+                                                 | 1 % ~01m 03s
|+                                                 | 2 % ~01m 02s
|++                                                | 3 % ~01m 01s
|++                                                | 4 % ~01m 01s
|+++                                               | 5 % ~01m 00s
|+++                                               | 6 % ~60s
|++++                                              | 7 % ~59s
|++++                                              | 8 % ~59s
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|+++++                                             | 10% ~57s
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  |++++++++++++++++++++++++++++++++++++++++++++++++++++ | 92% ~05s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++++| 93% ~04s
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  |++++++++++++++++++++++++++++++++++++++++++++++++++++++| 95% ~03s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++++| 96% ~03s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++++| 97% ~02s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++++| 98% ~01s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++++| 99% ~01s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++++| 100% elapsed=01m 04s
```

```
Calculating cluster 2
```

```
|                                                  | 0 % ~calculating
|+                                                 | 1 % ~59s
|+                                                 | 2 % ~59s
|++                                                | 3 % ~58s
|++                                                | 4 % ~57s
|+++                                               | 5 % ~57s
|+++                                               | 6 % ~56s
|++++                                              | 7 % ~56s
|++++                                              | 8 % ~56s
|+++++                                             | 9 % ~55s
|+++++                                             | 10% ~55s
|++++++                                            | 11% ~54s
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|++++++++++++++++++++++++++                        | 51% ~30s
|++++++++++++++++++++++++++                        | 52% ~29s
|+++++++++++++++++++++++++++                       | 53% ~28s
|+++++++++++++++++++++++++++                       | 54% ~28s
|++++++++++++++++++++++++++++                      | 55% ~27s
|++++++++++++++++++++++++++++                      | 56% ~27s
|+++++++++++++++++++++++++++++                     | 57% ~26s
|+++++++++++++++++++++++++++++                     | 58% ~26s
|++++++++++++++++++++++++++++++                    | 59% ~25s
|++++++++++++++++++++++++++++++                    | 60% ~24s
|+++++++++++++++++++++++++++++++                   | 61% ~24s
|+++++++++++++++++++++++++++++++                   | 62% ~23s
|++++++++++++++++++++++++++++++++                  | 63% ~23s
|++++++++++++++++++++++++++++++++                  | 64% ~22s
|+++++++++++++++++++++++++++++++++                 | 65% ~21s
|+++++++++++++++++++++++++++++++++                 | 66% ~21s
|++++++++++++++++++++++++++++++++++                | 67% ~20s
|++++++++++++++++++++++++++++++++++                | 68% ~19s
|+++++++++++++++++++++++++++++++++++               | 69% ~19s
|+++++++++++++++++++++++++++++++++++               | 70% ~18s
|++++++++++++++++++++++++++++++++++++              | 71% ~18s
|++++++++++++++++++++++++++++++++++++              | 72% ~17s
|+++++++++++++++++++++++++++++++++++++             | 73% ~16s
|+++++++++++++++++++++++++++++++++++++             | 74% ~16s
|++++++++++++++++++++++++++++++++++++++            | 75% ~15s
|++++++++++++++++++++++++++++++++++++++            | 76% ~15s
|+++++++++++++++++++++++++++++++++++++++           | 77% ~14s
|+++++++++++++++++++++++++++++++++++++++           | 78% ~13s
```

```
  |++++++++++++++++++++++++++++++++++++++++          | 79% ~13s
  |++++++++++++++++++++++++++++++++++++++++          | 80% ~12s
  |++++++++++++++++++++++++++++++++++++++++          | 81% ~12s
  |++++++++++++++++++++++++++++++++++++++++          | 82% ~11s
  |++++++++++++++++++++++++++++++++++++++++          | 83% ~10s
  |++++++++++++++++++++++++++++++++++++++++          | 84% ~10s
  |++++++++++++++++++++++++++++++++++++++++          | 85% ~09s
  |++++++++++++++++++++++++++++++++++++++++          | 86% ~09s
  |++++++++++++++++++++++++++++++++++++++++          | 87% ~08s
  |++++++++++++++++++++++++++++++++++++++++          | 88% ~07s
  |++++++++++++++++++++++++++++++++++++++++          | 89% ~07s
  |++++++++++++++++++++++++++++++++++++++++          | 90% ~06s
  |++++++++++++++++++++++++++++++++++++++++          | 91% ~05s
  |++++++++++++++++++++++++++++++++++++++++          | 92% ~05s
  |++++++++++++++++++++++++++++++++++++++++          | 93% ~04s
  |++++++++++++++++++++++++++++++++++++++++          | 94% ~04s
  |++++++++++++++++++++++++++++++++++++++++          | 95% ~03s
  |++++++++++++++++++++++++++++++++++++++++          | 96% ~02s
  |++++++++++++++++++++++++++++++++++++++++          | 97% ~02s
  |++++++++++++++++++++++++++++++++++++++++          | 98% ~01s
  |++++++++++++++++++++++++++++++++++++++++          | 99% ~01s
  |++++++++++++++++++++++++++++++++++++++++          | 100% elapsed=01m 01s
```

```
Calculating cluster 8
```

```
|                                                  | 0 % ~calculating
|+                                                 | 1 % ~01m 04s
|+                                                 | 2 % ~01m 02s
|++                                                | 3 % ~01m 02s
|++                                                | 4 % ~01m 01s
|+++                                               | 5 % ~01m 00s
|+++                                               | 6 % ~59s
|++++                                              | 7 % ~59s
|++++                                              | 8 % ~59s
|+++++                                             | 9 % ~58s
|+++++                                             | 10% ~57s
|++++++                                            | 11% ~57s
|++++++                                            | 12% ~56s
|+++++++                                           | 13% ~55s
|+++++++                                           | 14% ~55s
|++++++++                                          | 15% ~54s
|++++++++                                          | 16% ~53s
|+++++++++                                         | 17% ~53s
|+++++++++                                         | 18% ~52s
|++++++++++                                        | 19% ~51s
|++++++++++                                        | 20% ~51s
|+++++++++++                                       | 21% ~50s
|+++++++++++                                       | 22% ~49s
|++++++++++++                                      | 23% ~49s
|++++++++++++                                      | 24% ~48s
|++++++++++++                                      | 25% ~48s
|++++++++++++                                      | 26% ~47s
|+++++++++++++                                     | 27% ~47s
|+++++++++++++                                     | 28% ~46s
|+++++++++++++++                                   | 29% ~45s
|+++++++++++++++                                   | 30% ~45s
|++++++++++++++++                                  | 31% ~44s
|++++++++++++++++                                  | 32% ~43s
|+++++++++++++++++                                 | 33% ~43s
|+++++++++++++++++                                 | 34% ~42s
|++++++++++++++++++                                | 35% ~42s
|++++++++++++++++++                                | 36% ~41s
|+++++++++++++++++++                               | 37% ~40s
|+++++++++++++++++++                               | 38% ~40s
|++++++++++++++++++++                              | 39% ~39s
|++++++++++++++++++++                              | 40% ~38s
|+++++++++++++++++++++                             | 41% ~38s
|+++++++++++++++++++++                             | 42% ~37s
|++++++++++++++++++++++                            | 43% ~36s
|++++++++++++++++++++++                            | 44% ~36s
|+++++++++++++++++++++++                           | 45% ~35s
|+++++++++++++++++++++++                           | 46% ~34s
|++++++++++++++++++++++++                          | 47% ~34s
|++++++++++++++++++++++++                          | 48% ~33s
|+++++++++++++++++++++++++                         | 49% ~32s
|+++++++++++++++++++++++++                         | 50% ~32s
|++++++++++++++++++++++++++                        | 51% ~31s
|++++++++++++++++++++++++++                        | 52% ~31s
|+++++++++++++++++++++++++++                       | 53% ~30s
|+++++++++++++++++++++++++++                       | 54% ~29s
|++++++++++++++++++++++++++++                      | 55% ~29s
|++++++++++++++++++++++++++++                      | 56% ~28s
|+++++++++++++++++++++++++++++                     | 57% ~27s
|+++++++++++++++++++++++++++++                     | 58% ~27s
|++++++++++++++++++++++++++++++                    | 59% ~26s
|++++++++++++++++++++++++++++++                    | 60% ~26s
|+++++++++++++++++++++++++++++++                   | 61% ~25s
|+++++++++++++++++++++++++++++++                   | 62% ~24s
|++++++++++++++++++++++++++++++++                  | 63% ~24s
|++++++++++++++++++++++++++++++++                  | 64% ~23s
|+++++++++++++++++++++++++++++++++                 | 65% ~22s
|+++++++++++++++++++++++++++++++++                 | 66% ~22s
|++++++++++++++++++++++++++++++++++                | 67% ~21s
|++++++++++++++++++++++++++++++++++                | 68% ~20s
|+++++++++++++++++++++++++++++++++++               | 69% ~20s
|+++++++++++++++++++++++++++++++++++               | 70% ~19s
|++++++++++++++++++++++++++++++++++++              | 71% ~18s
|++++++++++++++++++++++++++++++++++++              | 72% ~18s
|+++++++++++++++++++++++++++++++++++++             | 73% ~17s
|+++++++++++++++++++++++++++++++++++++             | 74% ~17s
|++++++++++++++++++++++++++++++++++++++            | 75% ~16s
|++++++++++++++++++++++++++++++++++++++            | 76% ~15s
|+++++++++++++++++++++++++++++++++++++++           | 77% ~15s
|+++++++++++++++++++++++++++++++++++++++           | 78% ~14s
```

```
  |++++++++++++++++++++++++++++++++++++++++          | 79% ~13s
  |++++++++++++++++++++++++++++++++++++++++          | 80% ~13s
  |++++++++++++++++++++++++++++++++++++++++          | 81% ~12s
  |++++++++++++++++++++++++++++++++++++++++          | 82% ~11s
  |++++++++++++++++++++++++++++++++++++++++          | 83% ~11s
  |++++++++++++++++++++++++++++++++++++++++          | 84% ~10s
  |++++++++++++++++++++++++++++++++++++++++          | 85% ~10s
  |++++++++++++++++++++++++++++++++++++++++          | 86% ~09s
  |++++++++++++++++++++++++++++++++++++++++          | 87% ~08s
  |++++++++++++++++++++++++++++++++++++++++          | 88% ~08s
  |++++++++++++++++++++++++++++++++++++++++          | 89% ~07s
  |++++++++++++++++++++++++++++++++++++++++          | 90% ~06s
  |++++++++++++++++++++++++++++++++++++++++          | 91% ~06s
  |++++++++++++++++++++++++++++++++++++++++          | 92% ~05s
  |++++++++++++++++++++++++++++++++++++++++          | 93% ~04s
  |++++++++++++++++++++++++++++++++++++++++          | 94% ~04s
  |++++++++++++++++++++++++++++++++++++++++          | 95% ~03s
  |++++++++++++++++++++++++++++++++++++++++          | 96% ~03s
  |++++++++++++++++++++++++++++++++++++++++          | 97% ~02s
  |++++++++++++++++++++++++++++++++++++++++          | 98% ~01s
  |++++++++++++++++++++++++++++++++++++++++          | 99% ~01s
  |++++++++++++++++++++++++++++++++++++++++          | 100% elapsed=01m 03s
```

```
Calculating cluster 11
```

```
|                                                  | 0 % ~calculating
|+                                                 | 1 % ~59s
|+                                                 | 2 % ~59s
|++                                                | 3 % ~58s
|++                                                | 4 % ~58s
|+++                                               | 5 % ~58s
|+++                                               | 6 % ~57s
|++++                                              | 7 % ~57s
|++++                                              | 8 % ~56s
|+++++                                             | 9 % ~56s
|+++++                                             | 10% ~55s
|++++++                                            | 11% ~55s
|++++++                                            | 12% ~54s
|+++++++                                           | 13% ~53s
|+++++++                                           | 14% ~53s
|++++++++                                          | 15% ~52s
|++++++++                                          | 16% ~52s
|+++++++++                                         | 17% ~51s
|+++++++++                                         | 18% ~50s
|++++++++++                                        | 19% ~50s
|++++++++++                                        | 20% ~49s
|+++++++++++                                       | 21% ~49s
|+++++++++++                                       | 22% ~48s
|++++++++++++                                      | 23% ~47s
|++++++++++++                                      | 24% ~47s
|+++++++++++++                                     | 25% ~46s
|+++++++++++++                                     | 26% ~45s
|++++++++++++++                                    | 27% ~45s
|++++++++++++++                                    | 28% ~44s
|+++++++++++++++                                   | 29% ~44s
|+++++++++++++++                                   | 30% ~43s
|++++++++++++++++                                  | 31% ~42s
|++++++++++++++++                                  | 32% ~42s
|+++++++++++++++++                                 | 33% ~41s
|+++++++++++++++++                                 | 34% ~41s
|++++++++++++++++++                                | 35% ~40s
|++++++++++++++++++                                | 36% ~39s
|+++++++++++++++++++                               | 37% ~39s
|+++++++++++++++++++                               | 38% ~38s
|++++++++++++++++++++                              | 39% ~37s
|++++++++++++++++++++                              | 40% ~37s
|+++++++++++++++++++++                             | 41% ~36s
|+++++++++++++++++++++                             | 42% ~36s
|++++++++++++++++++++++                            | 43% ~35s
|++++++++++++++++++++++                            | 44% ~34s
|+++++++++++++++++++++++                           | 45% ~34s
|+++++++++++++++++++++++                           | 46% ~33s
|++++++++++++++++++++++++                          | 47% ~33s
|++++++++++++++++++++++++                          | 48% ~32s
|+++++++++++++++++++++++++                         | 49% ~31s
|+++++++++++++++++++++++++                         | 50% ~31s
|++++++++++++++++++++++++++                        | 51% ~30s
|++++++++++++++++++++++++++                        | 52% ~30s
|+++++++++++++++++++++++++++                       | 53% ~29s
|+++++++++++++++++++++++++++                       | 54% ~28s
|++++++++++++++++++++++++++++                      | 55% ~28s
|++++++++++++++++++++++++++++                      | 56% ~27s
|+++++++++++++++++++++++++++++                     | 57% ~27s
|+++++++++++++++++++++++++++++                     | 58% ~26s
|++++++++++++++++++++++++++++++                    | 59% ~25s
|++++++++++++++++++++++++++++++                    | 60% ~25s
|+++++++++++++++++++++++++++++++                   | 61% ~24s
|+++++++++++++++++++++++++++++++                   | 62% ~23s
|++++++++++++++++++++++++++++++++                  | 63% ~23s
|++++++++++++++++++++++++++++++++                  | 64% ~22s
|+++++++++++++++++++++++++++++++++                 | 65% ~22s
|+++++++++++++++++++++++++++++++++                 | 66% ~21s
|++++++++++++++++++++++++++++++++++                | 67% ~20s
|++++++++++++++++++++++++++++++++++                | 68% ~20s
|+++++++++++++++++++++++++++++++++++               | 69% ~19s
|+++++++++++++++++++++++++++++++++++               | 70% ~19s
|++++++++++++++++++++++++++++++++++++              | 71% ~18s
|++++++++++++++++++++++++++++++++++++              | 72% ~17s
|+++++++++++++++++++++++++++++++++++++             | 73% ~17s
|+++++++++++++++++++++++++++++++++++++             | 74% ~16s
|++++++++++++++++++++++++++++++++++++++            | 75% ~15s
|++++++++++++++++++++++++++++++++++++++            | 76% ~15s
|+++++++++++++++++++++++++++++++++++++++           | 77% ~14s
|+++++++++++++++++++++++++++++++++++++++           | 78% ~14s
```

```
  |+++++++++++++++++++++++++++++++++++++++          | 79% ~13s
  |+++++++++++++++++++++++++++++++++++++++          | 80% ~12s
  |+++++++++++++++++++++++++++++++++++++++++         | 81% ~12s
  |+++++++++++++++++++++++++++++++++++++++++         | 82% ~11s
  |+++++++++++++++++++++++++++++++++++++++++++       | 83% ~10s
  |+++++++++++++++++++++++++++++++++++++++++++       | 84% ~10s
  |+++++++++++++++++++++++++++++++++++++++++++++      | 85% ~09s
  |+++++++++++++++++++++++++++++++++++++++++++++      | 86% ~09s
  |+++++++++++++++++++++++++++++++++++++++++++++++    | 87% ~08s
  |+++++++++++++++++++++++++++++++++++++++++++++++    | 88% ~07s
  |+++++++++++++++++++++++++++++++++++++++++++++++++  | 89% ~07s
  |+++++++++++++++++++++++++++++++++++++++++++++++++  | 90% ~06s
  |+++++++++++++++++++++++++++++++++++++++++++++++++++ | 91% ~06s
  |+++++++++++++++++++++++++++++++++++++++++++++++++++ | 92% ~05s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 93% ~04s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 94% ~04s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 95% ~03s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 96% ~02s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 97% ~02s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 98% ~01s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 99% ~01s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 100% elapsed=01m 01s
```

```
Calculating cluster 10
```

```
|                                                  | 0 % ~calculating
|+                                                 | 1 % ~01m 14s
|++                                                | 2 % ~01m 14s
|++                                                | 3 % ~01m 14s
|+++                                               | 4 % ~01m 13s
|+++                                               | 5 % ~01m 12s
|++++                                              | 6 % ~01m 11s
|++++                                              | 7 % ~01m 11s
|+++++                                             | 8 % ~01m 11s
|+++++                                             | 9 % ~01m 10s
|++++++                                            | 10% ~01m 09s
|++++++                                            | 11% ~01m 08s
|+++++++                                           | 12% ~01m 07s
|+++++++                                           | 13% ~01m 06s
|++++++++                                          | 14% ~01m 06s
|++++++++                                          | 15% ~01m 05s
|+++++++++                                         | 16% ~01m 04s
|+++++++++                                         | 17% ~01m 03s
|++++++++++                                        | 18% ~01m 02s
|++++++++++                                        | 19% ~01m 02s
|+++++++++++                                       | 20% ~01m 01s
|+++++++++++                                       | 21% ~01m 00s
|++++++++++++                                      | 22% ~59s
|++++++++++++                                      | 23% ~58s
|++++++++++++++                                    | 24% ~58s
|++++++++++++++                                    | 25% ~57s
|++++++++++++++                                    | 26% ~56s
|++++++++++++++                                    | 27% ~55s
|+++++++++++++++                                   | 28% ~55s
|+++++++++++++++                                   | 29% ~54s
|++++++++++++++++                                  | 30% ~53s
|++++++++++++++++                                  | 31% ~53s
|++++++++++++++++++                                | 32% ~52s
|++++++++++++++++++                                | 33% ~51s
|+++++++++++++++++++                               | 34% ~50s
|+++++++++++++++++++                               | 35% ~50s
|++++++++++++++++++++                              | 36% ~49s
|++++++++++++++++++++                              | 37% ~48s
|+++++++++++++++++++++                             | 38% ~47s
|+++++++++++++++++++++                             | 39% ~47s
|++++++++++++++++++++++                            | 40% ~46s
|++++++++++++++++++++++                            | 41% ~45s
|+++++++++++++++++++++++                           | 42% ~44s
|+++++++++++++++++++++++                           | 43% ~43s
|++++++++++++++++++++++++                          | 44% ~43s
|++++++++++++++++++++++++                          | 45% ~42s
|+++++++++++++++++++++++++                         | 46% ~41s
|+++++++++++++++++++++++++                         | 47% ~40s
|++++++++++++++++++++++++++                        | 48% ~39s
|++++++++++++++++++++++++++                        | 49% ~39s
|+++++++++++++++++++++++++++                       | 51% ~38s
|+++++++++++++++++++++++++++                       | 52% ~37s
|++++++++++++++++++++++++++++                      | 53% ~36s
|++++++++++++++++++++++++++++                      | 54% ~36s
|++++++++++++++++++++++++++++                      | 55% ~35s
|+++++++++++++++++++++++++++++                     | 56% ~34s
|+++++++++++++++++++++++++++++                     | 57% ~33s
|++++++++++++++++++++++++++++++                    | 58% ~32s
|++++++++++++++++++++++++++++++                    | 59% ~32s
|+++++++++++++++++++++++++++++++                   | 60% ~31s
|+++++++++++++++++++++++++++++++                   | 61% ~30s
|++++++++++++++++++++++++++++++++                  | 62% ~29s
|++++++++++++++++++++++++++++++++                  | 63% ~29s
|+++++++++++++++++++++++++++++++++                 | 64% ~28s
|+++++++++++++++++++++++++++++++++                 | 65% ~27s
|++++++++++++++++++++++++++++++++++                | 66% ~26s
|++++++++++++++++++++++++++++++++++                | 67% ~25s
|+++++++++++++++++++++++++++++++++++               | 68% ~25s
|+++++++++++++++++++++++++++++++++++               | 69% ~24s
|++++++++++++++++++++++++++++++++++++              | 70% ~23s
|++++++++++++++++++++++++++++++++++++              | 71% ~22s
|+++++++++++++++++++++++++++++++++++++             | 72% ~22s
|+++++++++++++++++++++++++++++++++++++             | 73% ~21s
|++++++++++++++++++++++++++++++++++++++            | 74% ~20s
|++++++++++++++++++++++++++++++++++++++            | 75% ~19s
|+++++++++++++++++++++++++++++++++++++++           | 76% ~19s
|+++++++++++++++++++++++++++++++++++++++           | 77% ~18s
|++++++++++++++++++++++++++++++++++++++++          | 78% ~17s
|++++++++++++++++++++++++++++++++++++++++          | 79% ~16s
```

```
  |+++++++++++++++++++++++++++++++++++++++          | 80% ~15s
  |++++++++++++++++++++++++++++++++++++++++         | 81% ~15s
  |+++++++++++++++++++++++++++++++++++++++++        | 82% ~14s
  |++++++++++++++++++++++++++++++++++++++++++       | 83% ~13s
  |+++++++++++++++++++++++++++++++++++++++++++      | 84% ~12s
  |++++++++++++++++++++++++++++++++++++++++++++     | 85% ~12s
  |+++++++++++++++++++++++++++++++++++++++++++++    | 86% ~11s
  |++++++++++++++++++++++++++++++++++++++++++++++   | 87% ~10s
  |+++++++++++++++++++++++++++++++++++++++++++++++  | 88% ~09s
  |++++++++++++++++++++++++++++++++++++++++++++++++ | 89% ~08s
  |+++++++++++++++++++++++++++++++++++++++++++++++  | 90% ~08s
  |++++++++++++++++++++++++++++++++++++++++++++++++ | 91% ~07s
  |++++++++++++++++++++++++++++++++++++++++++++++++ | 92% ~06s
  |++++++++++++++++++++++++++++++++++++++++++++++++ | 93% ~05s
  |++++++++++++++++++++++++++++++++++++++++++++++++ | 94% ~05s
  |++++++++++++++++++++++++++++++++++++++++++++++++ | 95% ~04s
  |++++++++++++++++++++++++++++++++++++++++++++++++ | 96% ~03s
  |++++++++++++++++++++++++++++++++++++++++++++++++ | 97% ~02s
  |++++++++++++++++++++++++++++++++++++++++++++++++ | 98% ~02s
  |++++++++++++++++++++++++++++++++++++++++++++++++| 99% ~01s
  |++++++++++++++++++++++++++++++++++++++++++++++++| 100% elapsed=01m 16s
```

```
Calculating cluster 12
```

```
|                                                  | 0 % ~calculating
|+                                                 | 1 % ~01m 14s
|+                                                 | 2 % ~01m 14s
|++                                                | 3 % ~01m 13s
|++                                                | 4 % ~01m 12s
|+++                                               | 5 % ~01m 11s
|+++                                               | 6 % ~01m 11s
|++++                                              | 7 % ~01m 10s
|++++                                              | 8 % ~01m 10s
|+++++                                             | 9 % ~01m 09s
|+++++                                             | 10% ~01m 08s
|++++++                                            | 11% ~01m 08s
|++++++                                            | 12% ~01m 07s
|+++++++                                           | 13% ~01m 06s
|+++++++                                           | 14% ~01m 05s
|++++++++                                          | 15% ~01m 04s
|++++++++                                          | 16% ~01m 04s
|+++++++++                                         | 17% ~01m 03s
|+++++++++                                         | 18% ~01m 02s
|++++++++++                                        | 19% ~01m 02s
|++++++++++                                        | 20% ~01m 01s
|+++++++++++                                       | 21% ~01m 00s
|+++++++++++                                       | 22% ~59s
|++++++++++++                                      | 23% ~58s
|++++++++++++                                      | 24% ~58s
|++++++++++++                                      | 25% ~57s
|++++++++++++                                      | 26% ~56s
|+++++++++++++                                     | 27% ~55s
|+++++++++++++                                     | 28% ~55s
|+++++++++++++                                     | 29% ~54s
|+++++++++++++                                     | 30% ~53s
|++++++++++++++                                    | 31% ~52s
|++++++++++++++                                    | 32% ~52s
|++++++++++++++                                    | 33% ~51s
|++++++++++++++                                    | 34% ~50s
|+++++++++++++++                                   | 35% ~49s
|+++++++++++++++                                   | 36% ~49s
|+++++++++++++++                                   | 37% ~48s
|++++++++++++++++                                  | 38% ~47s
|++++++++++++++++                                  | 39% ~46s
|++++++++++++++++                                  | 40% ~45s
|++++++++++++++++                                  | 41% ~45s
|+++++++++++++++++                                 | 42% ~44s
|+++++++++++++++++                                 | 43% ~43s
|+++++++++++++++++                                 | 44% ~42s
|++++++++++++++++++                                | 45% ~42s
|++++++++++++++++++                                | 46% ~41s
|++++++++++++++++++                                | 47% ~40s
|+++++++++++++++++++                               | 48% ~40s
|+++++++++++++++++++                               | 49% ~39s
|+++++++++++++++++++                               | 50% ~38s
|++++++++++++++++++++                              | 51% ~37s
|++++++++++++++++++++                              | 52% ~36s
|++++++++++++++++++++                              | 53% ~36s
|++++++++++++++++++++                              | 54% ~35s
|+++++++++++++++++++++                             | 55% ~34s
|+++++++++++++++++++++                             | 56% ~33s
|+++++++++++++++++++++                             | 57% ~33s
|+++++++++++++++++++++                             | 58% ~32s
|++++++++++++++++++++++                            | 59% ~31s
|++++++++++++++++++++++                            | 60% ~30s
|++++++++++++++++++++++                            | 61% ~30s
|++++++++++++++++++++++                            | 62% ~29s
|+++++++++++++++++++++++                           | 63% ~28s
|+++++++++++++++++++++++                           | 64% ~27s
|+++++++++++++++++++++++                           | 65% ~27s
|+++++++++++++++++++++++                           | 66% ~26s
|++++++++++++++++++++++++                          | 67% ~25s
|++++++++++++++++++++++++                          | 68% ~24s
|++++++++++++++++++++++++                          | 69% ~24s
|++++++++++++++++++++++++                          | 70% ~23s
|+++++++++++++++++++++++++                         | 71% ~22s
|+++++++++++++++++++++++++                         | 72% ~21s
|+++++++++++++++++++++++++                         | 73% ~21s
|+++++++++++++++++++++++++                         | 74% ~20s
|++++++++++++++++++++++++++                        | 75% ~19s
|++++++++++++++++++++++++++                        | 76% ~18s
|++++++++++++++++++++++++++                        | 77% ~17s
|++++++++++++++++++++++++++                        | 78% ~17s
```

```
  |+++++++++++++++++++++++++++++++++++++++          | 79% ~16s
  |+++++++++++++++++++++++++++++++++++++++          | 80% ~15s
  |+++++++++++++++++++++++++++++++++++++++++         | 81% ~14s
  |+++++++++++++++++++++++++++++++++++++++++         | 82% ~14s
  |+++++++++++++++++++++++++++++++++++++++++++       | 83% ~13s
  |+++++++++++++++++++++++++++++++++++++++++++       | 84% ~12s
  |+++++++++++++++++++++++++++++++++++++++++++++      | 85% ~11s
  |+++++++++++++++++++++++++++++++++++++++++++++      | 86% ~11s
  |+++++++++++++++++++++++++++++++++++++++++++++++    | 87% ~10s
  |+++++++++++++++++++++++++++++++++++++++++++++++    | 88% ~09s
  |+++++++++++++++++++++++++++++++++++++++++++++++++  | 89% ~08s
  |+++++++++++++++++++++++++++++++++++++++++++++++++  | 90% ~08s
  |+++++++++++++++++++++++++++++++++++++++++++++++++++ | 91% ~07s
  |+++++++++++++++++++++++++++++++++++++++++++++++++++ | 92% ~06s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 93% ~05s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 94% ~05s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 95% ~04s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 96% ~03s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 97% ~02s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 98% ~02s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 99% ~01s
  |++++++++++++++++++++++++++++++++++++++++++++++++++++| 100% elapsed=01m 16s
```

```
Calculating cluster 7
```

```
|                                                  | 0 % ~calculating
|+                                                 | 1 % ~20s
|++                                                | 2 % ~19s
|++                                                | 3 % ~19s
|+++                                               | 4 % ~18s
|+++                                               | 5 % ~18s
|++++                                              | 6 % ~18s
|++++                                              | 7 % ~18s
|+++++                                             | 8 % ~18s
|+++++                                             | 9 % ~17s
|++++++                                            | 10% ~17s
|++++++                                            | 11% ~17s
|+++++++                                           | 12% ~17s
|+++++++                                           | 13% ~17s
|++++++++                                          | 14% ~17s
|++++++++                                          | 15% ~17s
|+++++++++                                         | 16% ~16s
|+++++++++                                         | 18% ~16s
|++++++++++                                        | 19% ~16s
|++++++++++                                        | 20% ~16s
|+++++++++++                                       | 21% ~15s
|+++++++++++                                       | 22% ~15s
|++++++++++++                                      | 23% ~15s
|++++++++++++                                      | 24% ~15s
|+++++++++++++                                     | 25% ~15s
|+++++++++++++                                     | 26% ~14s
|++++++++++++++                                    | 27% ~14s
|++++++++++++++                                    | 28% ~14s
|+++++++++++++++                                   | 29% ~14s
|+++++++++++++++                                   | 30% ~13s
|++++++++++++++++                                  | 31% ~13s
|++++++++++++++++                                  | 32% ~13s
|+++++++++++++++++                                 | 33% ~13s
|+++++++++++++++++                                 | 34% ~13s
|++++++++++++++++++                                | 35% ~12s
|++++++++++++++++++                                | 36% ~12s
|+++++++++++++++++++                               | 37% ~12s
|+++++++++++++++++++                               | 38% ~12s
|++++++++++++++++++++                              | 39% ~12s
|++++++++++++++++++++                              | 40% ~11s
|+++++++++++++++++++++                             | 41% ~11s
|+++++++++++++++++++++                             | 42% ~11s
|++++++++++++++++++++++                            | 43% ~11s
|++++++++++++++++++++++                            | 44% ~11s
|+++++++++++++++++++++++                           | 45% ~10s
|+++++++++++++++++++++++                           | 46% ~10s
|++++++++++++++++++++++++                          | 47% ~10s
|++++++++++++++++++++++++                          | 48% ~10s
|++++++++++++++++++++++++                          | 49% ~10s
|+++++++++++++++++++++++++                         | 51% ~09s
|+++++++++++++++++++++++++                         | 52% ~09s
|++++++++++++++++++++++++++                        | 53% ~09s
|++++++++++++++++++++++++++                        | 54% ~09s
|++++++++++++++++++++++++++                        | 55% ~09s
|+++++++++++++++++++++++++++                       | 56% ~08s
|+++++++++++++++++++++++++++                       | 57% ~08s
|+++++++++++++++++++++++++++                       | 58% ~08s
|++++++++++++++++++++++++++++                      | 59% ~08s
|++++++++++++++++++++++++++++                      | 60% ~08s
|+++++++++++++++++++++++++++++                     | 61% ~07s
|+++++++++++++++++++++++++++++                     | 62% ~07s
|+++++++++++++++++++++++++++++                     | 63% ~07s
|++++++++++++++++++++++++++++++                    | 64% ~07s
|++++++++++++++++++++++++++++++                    | 65% ~07s
|++++++++++++++++++++++++++++++                    | 66% ~06s
|+++++++++++++++++++++++++++++++                   | 67% ~06s
|+++++++++++++++++++++++++++++++                   | 68% ~06s
|++++++++++++++++++++++++++++++++                  | 69% ~06s
|++++++++++++++++++++++++++++++++                  | 70% ~06s
|++++++++++++++++++++++++++++++++                  | 71% ~05s
|+++++++++++++++++++++++++++++++++                 | 72% ~05s
|+++++++++++++++++++++++++++++++++                 | 73% ~05s
|++++++++++++++++++++++++++++++++++                | 74% ~05s
|++++++++++++++++++++++++++++++++++                | 75% ~05s
|+++++++++++++++++++++++++++++++++++               | 76% ~05s
|+++++++++++++++++++++++++++++++++++               | 77% ~04s
|++++++++++++++++++++++++++++++++++++              | 78% ~04s
|++++++++++++++++++++++++++++++++++++              | 79% ~04s
|+++++++++++++++++++++++++++++++++++++             | 80% ~04s
```

```
  |++++++++++++++++++++++++++++++++++++++++          | 81% ~04s
  |++++++++++++++++++++++++++++++++++++++++          | 82% ~03s
  |++++++++++++++++++++++++++++++++++++++++          | 84% ~03s
  |++++++++++++++++++++++++++++++++++++++++          | 85% ~03s
  |++++++++++++++++++++++++++++++++++++++++          | 86% ~03s
  |++++++++++++++++++++++++++++++++++++++++          | 87% ~03s
  |++++++++++++++++++++++++++++++++++++++++          | 88% ~02s
  |++++++++++++++++++++++++++++++++++++++++          | 89% ~02s
  |++++++++++++++++++++++++++++++++++++++++          | 90% ~02s
  |++++++++++++++++++++++++++++++++++++++++          | 91% ~02s
  |++++++++++++++++++++++++++++++++++++++++          | 92% ~02s
  |++++++++++++++++++++++++++++++++++++++++          | 93% ~01s
  |++++++++++++++++++++++++++++++++++++++++          | 94% ~01s
  |++++++++++++++++++++++++++++++++++++++++          | 95% ~01s
  |++++++++++++++++++++++++++++++++++++++++          | 96% ~01s
  |++++++++++++++++++++++++++++++++++++++++          | 97% ~01s
  |++++++++++++++++++++++++++++++++++++++++          | 98% ~00s
  |++++++++++++++++++++++++++++++++++++++++          | 99% ~00s
  |++++++++++++++++++++++++++++++++++++++++          | 100% elapsed=19s
```

```
Calculating cluster 3
```

```
|                                                  | 0 % ~calculating
|+                                                 | 1 % ~18s
|++                                                | 2 % ~18s
|++                                                | 3 % ~18s
|+++                                               | 4 % ~18s
|+++                                               | 5 % ~18s
|++++                                              | 6 % ~17s
|++++                                              | 7 % ~17s
|+++++                                             | 8 % ~17s
|+++++                                             | 9 % ~17s
|++++++                                            | 10% ~17s
|++++++                                            | 11% ~16s
|+++++++                                           | 12% ~16s
|+++++++                                           | 13% ~16s
|++++++++                                          | 14% ~16s
|++++++++                                          | 15% ~16s
|+++++++++                                         | 16% ~15s
|+++++++++                                         | 17% ~15s
|++++++++++                                        | 18% ~15s
|++++++++++                                        | 19% ~15s
|+++++++++++                                       | 20% ~15s
|+++++++++++                                       | 21% ~15s
|++++++++++++                                      | 22% ~14s
|++++++++++++                                      | 23% ~14s
|++++++++++++++                                    | 24% ~14s
|++++++++++++++                                    | 25% ~14s
|+++++++++++++++                                   | 26% ~14s
|+++++++++++++++                                   | 27% ~13s
|++++++++++++++++                                  | 28% ~13s
|++++++++++++++++                                  | 29% ~13s
|+++++++++++++++++                                 | 30% ~13s
|+++++++++++++++++                                 | 31% ~13s
|++++++++++++++++++                                | 32% ~12s
|++++++++++++++++++                                | 33% ~12s
|+++++++++++++++++++                               | 34% ~12s
|+++++++++++++++++++                               | 35% ~12s
|++++++++++++++++++++                              | 36% ~12s
|++++++++++++++++++++                              | 37% ~12s
|+++++++++++++++++++++                             | 38% ~11s
|+++++++++++++++++++++                             | 39% ~11s
|++++++++++++++++++++++                            | 40% ~11s
|++++++++++++++++++++++                            | 41% ~11s
|+++++++++++++++++++++++                           | 42% ~11s
|+++++++++++++++++++++++                           | 43% ~10s
|++++++++++++++++++++++++                          | 44% ~10s
|++++++++++++++++++++++++                          | 45% ~10s
|+++++++++++++++++++++++++                         | 46% ~10s
|+++++++++++++++++++++++++                         | 47% ~10s
|++++++++++++++++++++++++++                        | 48% ~09s
|++++++++++++++++++++++++++                        | 49% ~09s
|++++++++++++++++++++++++++                        | 51% ~09s
|++++++++++++++++++++++++++                        | 52% ~09s
|+++++++++++++++++++++++++++                       | 53% ~09s
|+++++++++++++++++++++++++++                       | 54% ~09s
|+++++++++++++++++++++++++++                       | 55% ~08s
|++++++++++++++++++++++++++++                      | 56% ~08s
|++++++++++++++++++++++++++++                      | 57% ~08s
|++++++++++++++++++++++++++++                      | 58% ~08s
|+++++++++++++++++++++++++++++                     | 59% ~08s
|+++++++++++++++++++++++++++++                     | 60% ~07s
|++++++++++++++++++++++++++++++                    | 61% ~07s
|++++++++++++++++++++++++++++++                    | 62% ~07s
|+++++++++++++++++++++++++++++++                   | 63% ~07s
|+++++++++++++++++++++++++++++++                   | 64% ~07s
|++++++++++++++++++++++++++++++++                  | 65% ~06s
|++++++++++++++++++++++++++++++++                  | 66% ~06s
|+++++++++++++++++++++++++++++++++                 | 67% ~06s
|+++++++++++++++++++++++++++++++++                 | 68% ~06s
|++++++++++++++++++++++++++++++++++                | 69% ~06s
|++++++++++++++++++++++++++++++++++                | 70% ~06s
|+++++++++++++++++++++++++++++++++++               | 71% ~05s
|+++++++++++++++++++++++++++++++++++               | 72% ~05s
|++++++++++++++++++++++++++++++++++++              | 73% ~05s
|++++++++++++++++++++++++++++++++++++              | 74% ~05s
|+++++++++++++++++++++++++++++++++++++             | 75% ~05s
|+++++++++++++++++++++++++++++++++++++             | 76% ~04s
|++++++++++++++++++++++++++++++++++++++            | 77% ~04s
|++++++++++++++++++++++++++++++++++++++            | 78% ~04s
|+++++++++++++++++++++++++++++++++++++++           | 79% ~04s
```

```
  |++++++++++++++++++++++++++++++++++++++++          | 80% ~04s
  |++++++++++++++++++++++++++++++++++++++++          | 81% ~04s
  |++++++++++++++++++++++++++++++++++++++++          | 82% ~03s
  |++++++++++++++++++++++++++++++++++++++++          | 83% ~03s
  |++++++++++++++++++++++++++++++++++++++++          | 84% ~03s
  |++++++++++++++++++++++++++++++++++++++++          | 85% ~03s
  |++++++++++++++++++++++++++++++++++++++++          | 86% ~03s
  |++++++++++++++++++++++++++++++++++++++++          | 87% ~02s
  |++++++++++++++++++++++++++++++++++++++++          | 88% ~02s
  |++++++++++++++++++++++++++++++++++++++++          | 89% ~02s
  |++++++++++++++++++++++++++++++++++++++++          | 90% ~02s
  |++++++++++++++++++++++++++++++++++++++++          | 91% ~02s
  |++++++++++++++++++++++++++++++++++++++++          | 92% ~01s
  |++++++++++++++++++++++++++++++++++++++++          | 93% ~01s
  |++++++++++++++++++++++++++++++++++++++++          | 94% ~01s
  |++++++++++++++++++++++++++++++++++++++++          | 95% ~01s
  |++++++++++++++++++++++++++++++++++++++++          | 96% ~01s
  |++++++++++++++++++++++++++++++++++++++++          | 97% ~01s
  |++++++++++++++++++++++++++++++++++++++++          | 98% ~00s
  |++++++++++++++++++++++++++++++++++++++++          | 99% ~00s
  |++++++++++++++++++++++++++++++++++++++++          | 100% elapsed=18s
```

```
Calculating cluster 6
```

```
|                                                  | 0 % ~calculating
|+                                                 | 1 % ~15s
|++                                                | 2 % ~15s
|++                                                | 3 % ~15s
|+++                                               | 4 % ~14s
|+++                                               | 5 % ~14s
|++++                                              | 6 % ~14s
|++++                                              | 7 % ~14s
|+++++                                             | 8 % ~14s
|+++++                                             | 9 % ~13s
|++++++                                            | 10% ~13s
|++++++                                            | 11% ~13s
|+++++++                                           | 12% ~13s
|+++++++                                           | 13% ~13s
|++++++++                                          | 14% ~13s
|++++++++                                          | 15% ~13s
|+++++++++                                         | 16% ~12s
|+++++++++                                         | 17% ~12s
|++++++++++                                        | 18% ~12s
|++++++++++                                        | 19% ~12s
|+++++++++++                                       | 20% ~12s
|+++++++++++                                       | 21% ~12s
|++++++++++++                                      | 22% ~12s
|++++++++++++                                      | 23% ~11s
|+++++++++++++                                     | 24% ~11s
|+++++++++++++                                     | 25% ~11s
|+++++++++++++                                     | 26% ~11s
|+++++++++++++                                     | 27% ~11s
|++++++++++++++                                    | 28% ~11s
|++++++++++++++                                    | 29% ~10s
|+++++++++++++++                                   | 30% ~10s
|+++++++++++++++                                   | 31% ~10s
|++++++++++++++++                                  | 32% ~10s
|++++++++++++++++                                  | 33% ~10s
|+++++++++++++++++                                 | 34% ~10s
|+++++++++++++++++                                 | 35% ~10s
|++++++++++++++++++                                | 36% ~09s
|++++++++++++++++++                                | 37% ~09s
|+++++++++++++++++++                               | 38% ~09s
|+++++++++++++++++++                               | 39% ~09s
|++++++++++++++++++++                              | 40% ~09s
|++++++++++++++++++++                              | 41% ~09s
|+++++++++++++++++++++                             | 42% ~09s
|+++++++++++++++++++++                             | 43% ~08s
|++++++++++++++++++++++                            | 44% ~08s
|++++++++++++++++++++++                            | 45% ~08s
|+++++++++++++++++++++++                           | 46% ~08s
|+++++++++++++++++++++++                           | 47% ~08s
|++++++++++++++++++++++++                          | 48% ~08s
|++++++++++++++++++++++++                          | 49% ~08s
|++++++++++++++++++++++++++                        | 51% ~07s
|++++++++++++++++++++++++++                        | 52% ~07s
|+++++++++++++++++++++++++++                       | 53% ~07s
|+++++++++++++++++++++++++++                       | 54% ~07s
|+++++++++++++++++++++++++++                       | 55% ~07s
|++++++++++++++++++++++++++++                      | 56% ~07s
|++++++++++++++++++++++++++++                      | 57% ~06s
|++++++++++++++++++++++++++++                      | 58% ~06s
|+++++++++++++++++++++++++++++                     | 59% ~06s
|+++++++++++++++++++++++++++++                     | 60% ~06s
|++++++++++++++++++++++++++++++                    | 61% ~06s
|++++++++++++++++++++++++++++++                    | 62% ~06s
|+++++++++++++++++++++++++++++++                   | 63% ~06s
|+++++++++++++++++++++++++++++++                   | 64% ~05s
|++++++++++++++++++++++++++++++++                  | 65% ~05s
|++++++++++++++++++++++++++++++++                  | 66% ~05s
|+++++++++++++++++++++++++++++++++                 | 67% ~05s
|+++++++++++++++++++++++++++++++++                 | 68% ~05s
|++++++++++++++++++++++++++++++++++                | 69% ~05s
|++++++++++++++++++++++++++++++++++                | 70% ~05s
|+++++++++++++++++++++++++++++++++++               | 71% ~04s
|+++++++++++++++++++++++++++++++++++               | 72% ~04s
|++++++++++++++++++++++++++++++++++++              | 73% ~04s
|++++++++++++++++++++++++++++++++++++              | 74% ~04s
|+++++++++++++++++++++++++++++++++++++             | 75% ~04s
|+++++++++++++++++++++++++++++++++++++             | 76% ~04s
|++++++++++++++++++++++++++++++++++++++            | 77% ~03s
|++++++++++++++++++++++++++++++++++++++            | 78% ~03s
|+++++++++++++++++++++++++++++++++++++++           | 79% ~03s
```

```
  |+++++++++++++++++++++++++++++++++++++++          | 80% ~03s
  |++++++++++++++++++++++++++++++++++++++++         | 81% ~03s
  |+++++++++++++++++++++++++++++++++++++++++        | 82% ~03s
  |++++++++++++++++++++++++++++++++++++++++++       | 83% ~03s
  |+++++++++++++++++++++++++++++++++++++++++++      | 84% ~02s
  |++++++++++++++++++++++++++++++++++++++++++++     | 85% ~02s
  |+++++++++++++++++++++++++++++++++++++++++++++    | 86% ~02s
  |++++++++++++++++++++++++++++++++++++++++++++++   | 87% ~02s
  |+++++++++++++++++++++++++++++++++++++++++++++++  | 88% ~02s
  |++++++++++++++++++++++++++++++++++++++++++++++++ | 89% ~02s
  |+++++++++++++++++++++++++++++++++++++++++++++++++| 90% ~02s
  |++++++++++++++++++++++++++++++++++++++++++++++++++| 91% ~01s
  |++++++++++++++++++++++++++++++++++++++++++++++++++| 92% ~01s
  |++++++++++++++++++++++++++++++++++++++++++++++++++| 93% ~01s
  |++++++++++++++++++++++++++++++++++++++++++++++++++| 94% ~01s
  |++++++++++++++++++++++++++++++++++++++++++++++++++| 95% ~01s
  |++++++++++++++++++++++++++++++++++++++++++++++++++| 96% ~01s
  |++++++++++++++++++++++++++++++++++++++++++++++++++| 97% ~00s
  |++++++++++++++++++++++++++++++++++++++++++++++++++| 98% ~00s
  |++++++++++++++++++++++++++++++++++++++++++++++++++| 99% ~00s
  |++++++++++++++++++++++++++++++++++++++++++++++++++| 100% elapsed=15s
```

Hide

```
# Add entrez column with entrez Ids of genes
markers$entrez <- mapIds(org.Hs.eg.db,
                         keys = markers$gene,
                         column = "ENTREZID",
                         keytype = "SYMBOL",
                         multiVals = "first")
```

```
'select()' returned 1:1 mapping between keys and columns
```

Hide

```
# Select the 15 best ranked genes
markers %>%
  group_by(cluster) %>%
  dplyr::filter(avg_log2FC >= 1 , p_val_adj <= 0.05) %>%
  slice_head(n = 15) %>%
  ungroup() -> top15
```

Hide

```
# Manually annotating clusters using top 15 DEG from each cluster with CellMarkers BD, using tool CellMarker_anno
tation (http://www.bio-bigdata.center/CellMarker_annotation.jsp)
```

Hide

```
markers %>%
  group_by(cluster) %>%
  dplyr::filter(avg_log2FC >= 1 , p_val_adj <= 0.05) %>%
  slice_head(n = 10) %>%
  ungroup() -> top10

object_subset <- ScaleData(object_subset, assay = "Spatial.008um", features = top10$gene)
```

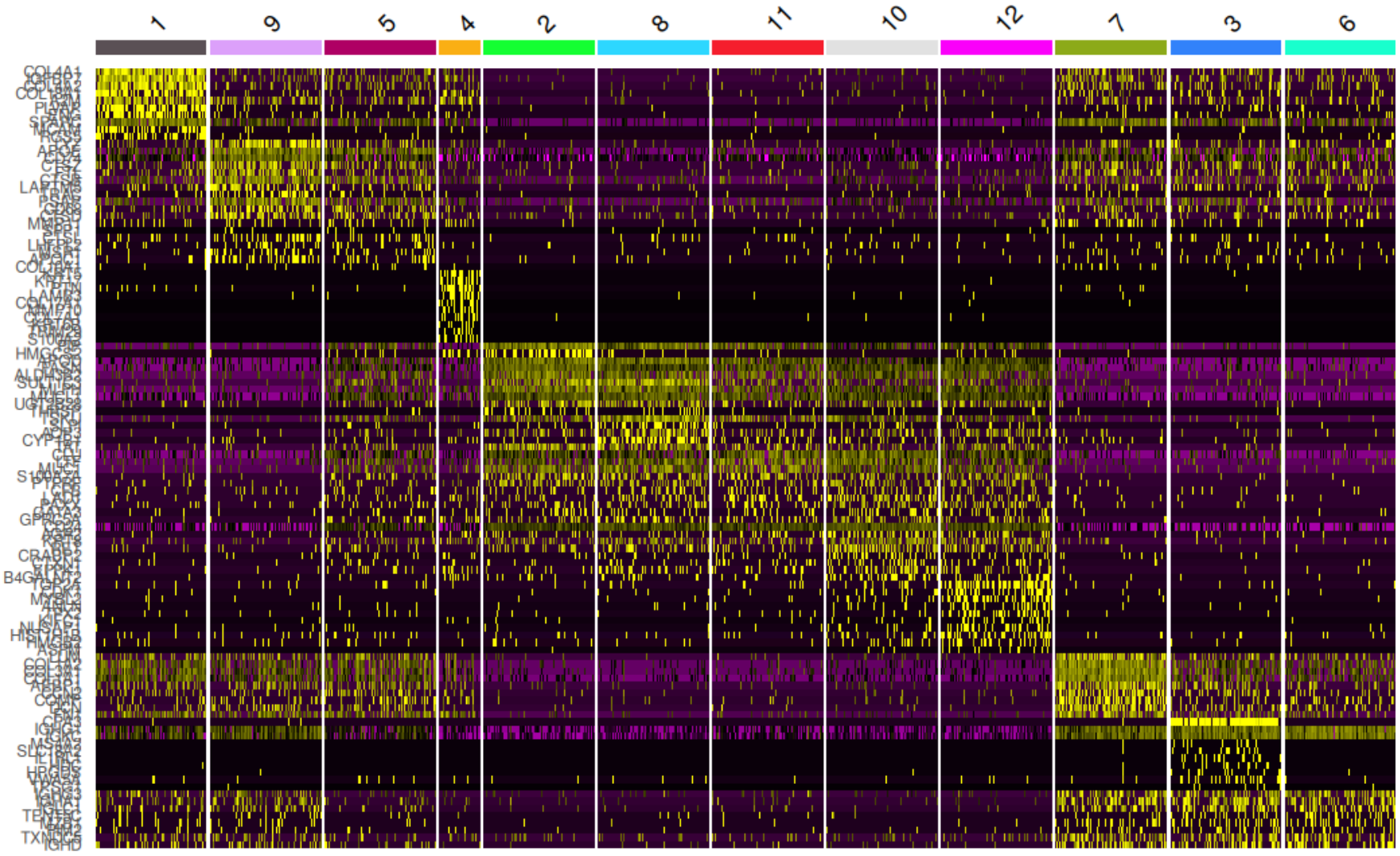
```
Centering and scaling data matrix

  |
  |
|   0%
  |
  |=============================================================================================================================
=============================================================| 100%
Warning: Different features in new layer data than already exists for scale.data
```

Hide

```
heatmap <- DoHeatmap(object_subset, assay = "Spatial.008um", features = top10$gene, size = 3, group.colors = colo
r_pal) + theme(axis.text = element_text(size = 5.5), legend.position = "none")
heatmap

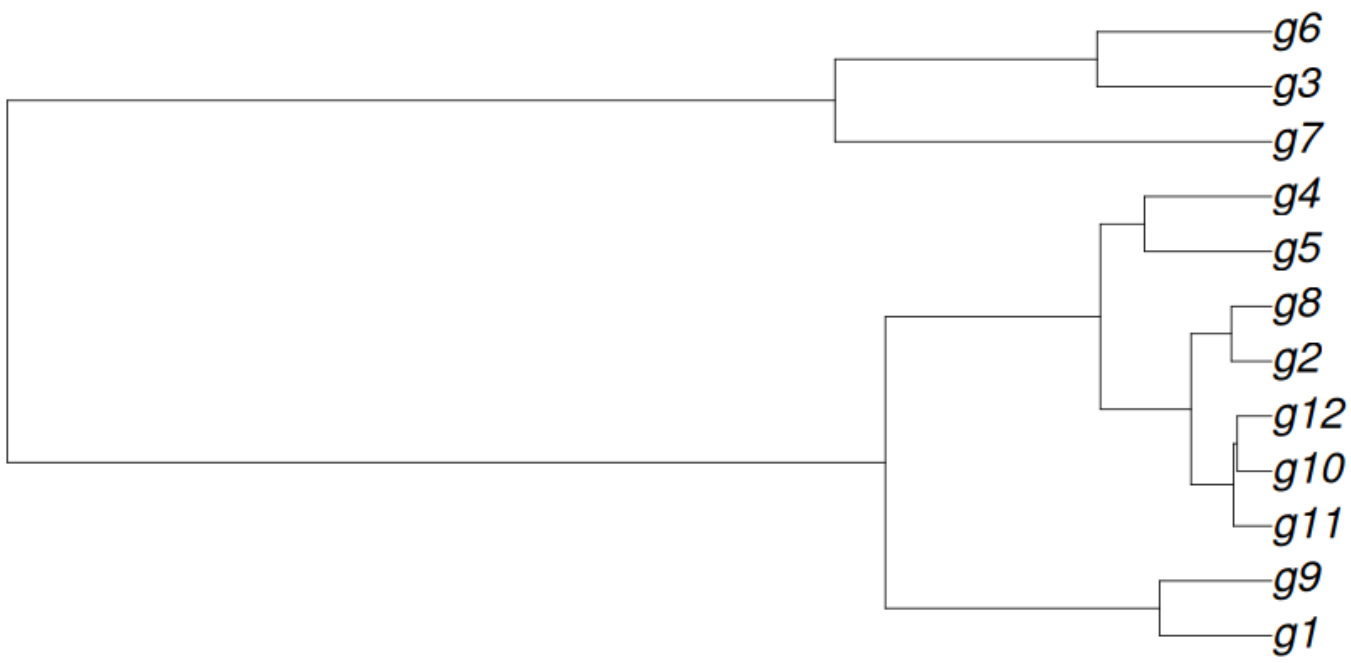

ggsave("heatmap.png", plot=heatmap, width=16, height=10, dpi =600)
```



Hide

```
# Build Tree only based on the top 10 genes per cluster
object_subset10 = BuildClusterTree(object_subset, features = top10$gene, reorder.numeric = T)

# Plot dendogram
data.tree <- Tool(object = object_subset10, slot = "BuildClusterTree")
ape::plot.phylo(x = data.tree, direction = "rightwards", edge.width=0.5)
```



Hide

```
# Assign cell types to metadata
clusters = object_filt@meta.data$seurat_cluster.projected

levels(clusters)
```

```
 [1] "1"  "2"  "3"  "4"  "5"  "6"  "7"  "8"  "9"  "10" "11" "12"
```

Hide

```
levels(clusters) = c("1.Célula madre hematopoyética/Pericito","2.Célula madre cancerosa","3.Mastocito/Célula plas
mática", "4.Célula basal/Célula epitelial basal","5.Panmacrófago/Macrófago M1","6.Célula plasmática/Célula B","7.
Miofibroblasto/Pericito","8.Célula progenitora epitelial/Célula B reguladora B10","9.Panmacrófago/Fibroblasto aso
c. a cáncer","10.Célula progenitora epitelial/Fibroblasto asoc. a cáncer","11.Fibroblasto asoc. a cáncer/Célula e
pitelial luminal","12.Célula progenitora luminal")

object_filt@meta.data$seurat_cluster.projected = clusters

# Set color palette
color_pal <- Seurat::DiscretePalette(n = length(unique(object_filt$seurat_cluster.projected)),
                                     palette = "polychrome")
names(color_pal) <- sort(unique(object_filt$seurat_cluster.projected))
```

Hide

```
# Review the levels
levels(Idents(object_filt))
```

```
 [1] "1"  "2"  "3"  "4"  "5"  "6"  "7"  "8"  "9"  "10" "11" "12"
```

Hide

```
head(object_filt@meta.data)
```

	orig.ident <chr>	nCount_Spatial.008um <dbl>	nFeature_Spatial.008um <int>	percent.mt <dbl>
s_008um_00269_00526-1	s	1479	1023	5.814740
s_008um_00260_00253-1	s	971	719	7.106076
s_008um_00433_00599-1	s	527	214	1.328273
s_008um_00266_00304-1	s	749	547	6.275033
s_008um_00359_00037-1	s	999	721	8.608609
s_008um_00469_00254-1	s	606	470	10.231023

6 rows | 1-5 of 9 columns

Hide

```
levels(object_filt@meta.data$seurat_cluster.projected)
```

```
 [1] "1.Célula madre hematopoyética/Pericito"                    "2.Célula madre cancerosa"
 [3] "3.Mastocito/Célula plasmática"                             "4.Célula basal/Célula epitelial basal"
 [5] "5.Panmacrófago/Macrófago M1"                               "6.Célula plasmática/Célula B"
 [7] "7.Miofibroblasto/Pericito"                                 "8.Célula progenitora epitelial/Célula B regula
dora B10"
 [9] "9.Panmacrófago/Fibroblasto asoc. a cáncer"                 "10.Célula progenitora epitelial/Fibroblasto as
oc. a cáncer"
[11] "11.Fibroblasto asoc. a cáncer/Célula epitelial luminal"    "12.Célula progenitora luminal"
```

Hide

```
# Assign cell types to object
levels(Idents(object_filt))
```

```
 [1] "1"  "2"  "3"  "4"  "5"  "6"  "7"  "8"  "9"  "10" "11" "12"
```

Hide

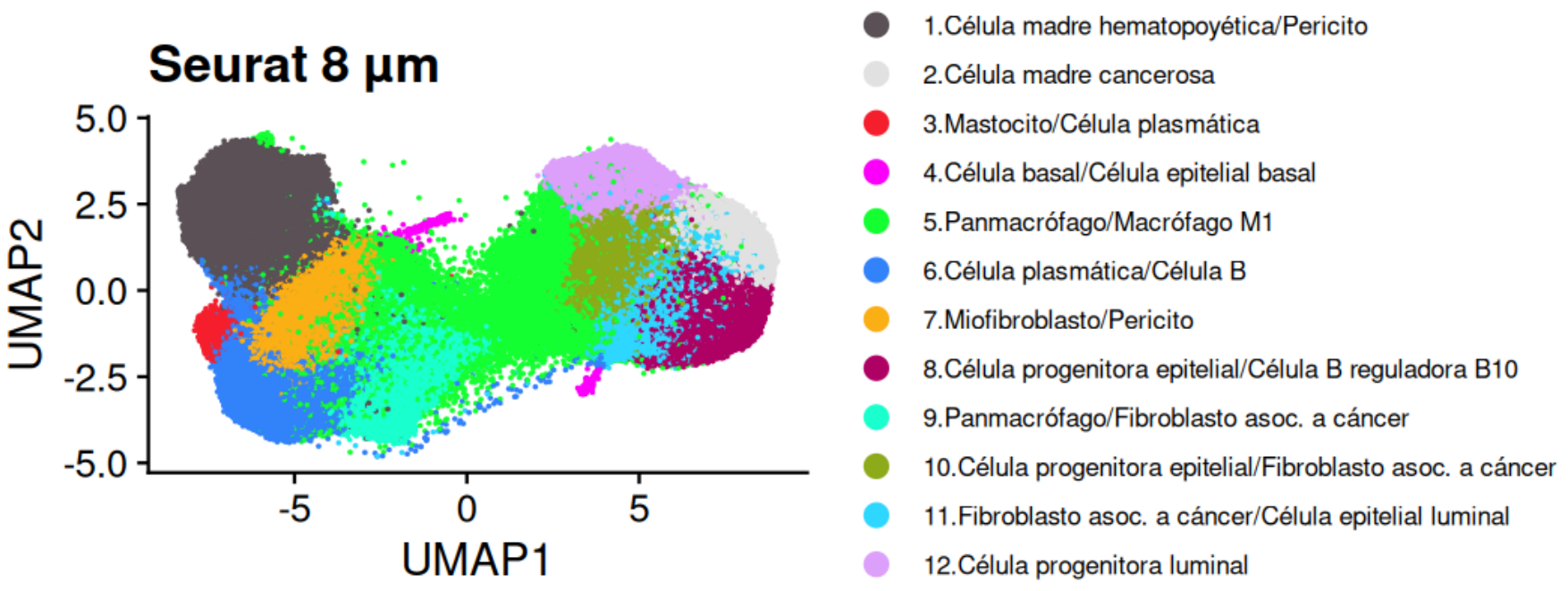
```
# Create new cluster names with cell types
new_cluster_names = c(
  "1" = "1.Célula madre hematopoyética/Pericito",
  "2" = "2.Célula madre cancerosa",
  "3" = "3.Mastocito/Célula plasmática",
  "4" = "4.Célula basal/Célula epitelial basal",
  "5" = "5.Panmacrófago/Macrófago M1",
  "6" = "6.Célula plasmática/Célula B",
  "7" = "7.Miofibroblasto/Pericito",
  "8" = "8.Célula progenitora epitelial/Célula B reguladora B10",
  "9" = "9.Panmacrófago/Fibroblasto asoc. a cáncer",
  "10" = "10.Célula progenitora epitelial/Fibroblasto asoc. a cáncer",
  "11" = "11.Fibroblasto asoc. a cáncer/Célula epitelial luminal",
  "12" = "12.Célula progenitora luminal"
)

# Assign cell types to clusters
names(new_cluster_names) = levels(object_filt)
object_filt = RenameIdents(object_filt, new_cluster_names)
levels(Idents(object_filt))
```

```
 [1] "1.Célula madre hematopoyética/Pericito"                  "2.Célula madre cancerosa"
 [3] "3.Mastocito/Célula plasmática"                           "4.Célula basal/Célula epitelial basal"
 [5] "5.Panmacrófago/Macrófago M1"                             "6.Célula plasmática/Célula B"
 [7] "7.Miofibroblasto/Pericito"                               "8.Célula progenitora epitelial/Célula B regula
dora B10"
 [9] "9.Panmacrófago/Fibroblasto asoc. a cáncer"               "10.Célula progenitora epitelial/Fibroblasto as
oc. a cáncer"
[11] "11.Fibroblasto asoc. a cáncer/Célula epitelial luminal"  "12.Célula progenitora luminal"
```

Hide

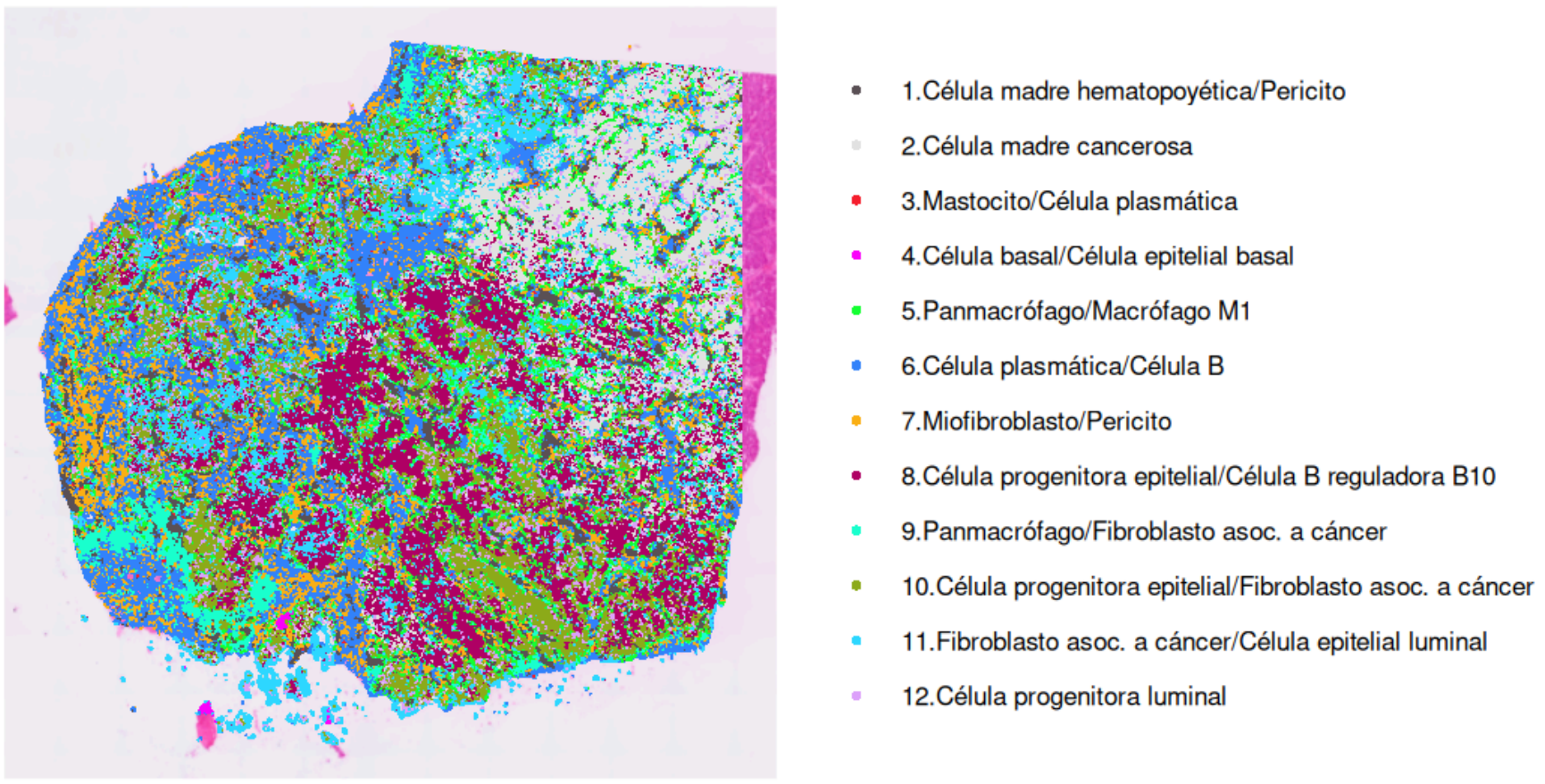
```
# Plot UMAP
umap_cells = DimPlot(object_filt, reduction = "full.umap.sketch", label = TRUE, raster=F, pt.size = 0.02, label.s
ize = 0, cols = color_pal)
umap_cells + coord_fixed(ratio = 1) +
  ggtitle("Seurat 8 µm") +
  xlab("UMAP1")+
  ylab("UMAP2")+
  theme(legend.text = element_text(size=8))
```



Hide

```
#  Visualize the unsupervised clusters based on their spatial location.
image_seurat_clusters2 <- SpatialDimPlot(object_filt,
                                         group.by = 'seurat_cluster.projected',
                                         pt.size.factor = 6, cols = color_pal)

image_seurat_clusters2  + ggtitle("Seurat 8 µm") +
  theme(legend.title = element_blank(), legend.text = element_text(size=8))
```



Hide

```
# Prepare objects to perform GO

# Define gene universe, all genes in markers
universe_genes = unique(na.omit(markers$entrez))

# Filter markers avg_log2FC > 1 and p_val_adj < 0.05
filtered_markers = markers %>%
  group_by(cluster) %>%
  dplyr::filter(avg_log2FC >= 1, p_val_adj <= 0.05)
```

Hide

```
# Run goana for each cluster with top 15 markers
go_results_list15 = list()

clusters15 = unique(top15$cluster)

for (cl in clusters15) {
  entrez_cl15 = top15 %>%
    filter(cluster == cl) %>%
    pull(entrez)

  go_r15 = goana(entrez_cl15, universe = universe_genes, species = "Hs")
  go_results_list15[[as.character(cl)]] = topGO(go_r15, number = 10, ontology = "BP")
  print(names(go_results_list15[cl]))
  print(go_results_list15[[cl]])
}
```

```
[1] "1"
```

	Term <chr>	O.. <chr>	N <dbl>	DE <dbl>	P.DE <dbl>
GO:0001525	angiogenesis	BP	404	10	1.089861e-11
GO:0048514	blood vessel morphogenesis	BP	468	10	4.685361e-11
GO:0001568	blood vessel development	BP	535	10	1.755911e-10

	Term	O..	N	DE	P.DE
	<chr>	<chr>	<dbl>	<dbl>	<dbl>
GO:0001944	vasculature development	BP	557	10	2.611068e-10
GO:0035239	tube morphogenesis	BP	633	10	9.165101e-10
GO:0035295	tube development	BP	774	10	6.518964e-09
GO:0072359	circulatory system development	BP	800	10	8.984225e-09
GO:0048646	anatomical structure formation involved in morphogenesis	BP	803	10	9.316414e-09
GO:0003094	glomerular filtration	BP	22	3	3.174068e-06
GO:0097205	renal filtration	BP	25	3	4.728779e-06

1-10 of 10 rows

```
[1] "9"
```

	Term	O..	N	D..
	<chr>	<chr>	<dbl>	<dbl>
GO:0009605	response to external stimulus	BP	1475	10
GO:0006952	defense response	BP	1138	9
GO:0009607	response to biotic stimulus	BP	996	8
GO:2000646	positive regulation of receptor catabolic process	BP	6	2
GO:0044419	biological process involved in interspecies interaction between organisms	BP	1096	8
GO:0002682	regulation of immune system process	BP	1102	8
GO:0050866	negative regulation of cell activation	BP	151	4
GO:0006955	immune response	BP	1154	8
GO:0002604	regulation of dendritic cell antigen processing and presentation	BP	10	2
GO:2000644	regulation of receptor catabolic process	BP	10	2

1-10 of 10 rows | 1-5 of 5 columns

```
[1] "5"
```

GO:0019886
GO:0042157
GO:0002495
GO:0002504
GO:0019882
GO:0002478
GO:0006898
GO:0055094
GO:0019884
GO:0034381

1-10 of 10 rows | 1-1 of 5 columns

```
[1] "4"
```

	Term	O...	N	DE	P.DE
	<chr>	<chr>	<dbl>	<dbl>	<dbl>
GO:0008544	epidermis development	BP	225	6	3.058028e-07
GO:0031424	keratinization	BP	19	3	2.002136e-06
GO:0045109	intermediate filament organization	BP	22	3	3.174068e-06
GO:0045104	intermediate filament cytoskeleton organization	BP	38	3	1.715938e-05
GO:0045103	intermediate filament-based process	BP	38	3	1.715938e-05
GO:0009888	tissue development	BP	1291	8	1.111907e-04

	Term <chr>	O… <chr>	N <dbl>	DE <dbl>	P.DE <dbl>
GO:0030216	keratinocyte differentiation	BP	86	3	2.000826e-04
GO:0045229	external encapsulating structure organization	BP	239	4	2.520534e-04
GO:0030198	extracellular matrix organization	BP	239	4	2.520534e-04
GO:0043062	extracellular structure organization	BP	239	4	2.520534e-04

1-10 of 10 rows

```
[1] "2"
```

	Term <chr>	Ont <chr>	N <dbl>	DE <dbl>	P.DE <dbl>
GO:0052697	xenobiotic glucuronidation	BP	2	2	1.524715e-06
GO:0006629	lipid metabolic process	BP	856	8	2.694781e-06
GO:0052695	cellular glucuronidation	BP	4	2	9.134897e-06
GO:0044281	small molecule metabolic process	BP	1171	8	2.822794e-05
GO:0006805	xenobiotic metabolic process	BP	54	3	3.997288e-05
GO:0019585	glucuronate metabolic process	BP	8	2	4.250479e-05
GO:0006063	uronic acid metabolic process	BP	8	2	4.250479e-05
GO:0008202	steroid metabolic process	BP	190	4	7.737566e-05
GO:0008210	estrogen metabolic process	BP	13	2	1.179734e-04
GO:0009410	response to xenobiotic stimulus	BP	257	4	2.485447e-04

1-10 of 10 rows

```
[1] "8"
```

	Term <chr>	O.. <chr>	N <dbl>	DE <dbl>
GO:0071635	negative regulation of transforming growth factor beta production	BP	10	2
GO:0016125	sterol metabolic process	BP	105	3
GO:0071634	regulation of transforming growth factor beta production	BP	28	2
GO:0071548	response to dexamethasone	BP	28	2
GO:1901615	organic hydroxy compound metabolic process	BP	310	4
GO:0071604	transforming growth factor beta production	BP	30	2
GO:0042221	response to chemical	BP	2219	9
GO:0071320	cellular response to cAMP	BP	33	2
GO:0015850	organic hydroxy compound transport	BP	154	3
GO:0032597	B cell receptor transport into membrane raft	BP	1	1

1-10 of 10 rows | 1-5 of 5 columns

```
[1] "11"
```

GO:0006959
GO:0048799
GO:1902230
GO:0043066
GO:0043069
GO:1902229
GO:0006956
GO:0002253
GO:0098630

GO:0042710

1-10 of 10 rows | 1-1 of 5 columns

```
[1] "10"
```

	Term <chr>	Ont <chr>	N <dbl>	DE <dbl>	P.DE <dbl>
GO:0009888	tissue development	BP	1291	9	1.123814e-05
GO:0017038	protein import	BP	11	2	9.607215e-05
GO:0007155	cell adhesion	BP	1021	7	2.001726e-04
GO:1902533	positive regulation of intracellular signal transduction	BP	772	6	3.510740e-04
GO:0048799	animal organ maturation	BP	21	2	3.639223e-04
GO:0060429	epithelium development	BP	785	6	3.844648e-04
GO:0050793	regulation of developmental process	BP	1646	8	6.217095e-04
GO:0051094	positive regulation of developmental process	BP	904	6	8.225564e-04
GO:0030155	regulation of cell adhesion	BP	589	5	8.551243e-04
GO:0009653	anatomical structure morphogenesis	BP	1744	8	9.273109e-04

1-10 of 10 rows

```
[1] "12"
```

	Term <chr>	Ont <chr>	N <dbl>	DE <dbl>	P.DE <dbl>
GO:0000280	nuclear division	BP	317	9	8.407861e-12
GO:0048285	organelle fission	BP	354	9	2.269782e-11
GO:0098813	nuclear chromosome segregation	BP	243	8	6.242417e-11
GO:0022402	cell cycle process	BP	972	11	1.729028e-10
GO:0051276	chromosome organization	BP	467	9	2.709723e-10
GO:0000819	sister chromatid segregation	BP	193	7	7.552913e-10
GO:0007059	chromosome segregation	BP	336	8	8.289123e-10
GO:0007049	cell cycle	BP	1223	11	2.089808e-09
GO:0140014	mitotic nuclear division	BP	228	7	2.425180e-09
GO:1903047	mitotic cell cycle process	BP	607	9	2.783825e-09

1-10 of 10 rows

```
[1] "7"
```

	Term <chr>	Ont <chr>	N <dbl>	DE <dbl>	P.DE <dbl>
GO:0030199	collagen fibril organization	BP	62	7	8.298952e-13
GO:0001568	blood vessel development	BP	535	10	1.755911e-10
GO:0001944	vasculature development	BP	557	10	2.611068e-10
GO:0072359	circulatory system development	BP	800	10	8.984225e-09
GO:0045229	external encapsulating structure organization	BP	239	7	1.216970e-08
GO:0030198	extracellular matrix organization	BP	239	7	1.216970e-08
GO:0043062	extracellular structure organization	BP	239	7	1.216970e-08
GO:0048514	blood vessel morphogenesis	BP	468	8	5.271343e-08
GO:0071604	transforming growth factor beta production	BP	30	4	6.172500e-08
GO:0018149	peptide cross-linking	BP	9	3	1.749966e-07

1-10 of 10 rows

```
[1] "3"
```

GO:0050853
GO:0002443
GO:0006958
GO:0019731
GO:0002460
GO:0016064
GO:0006956
GO:0019724
GO:0002455
GO:0002252

1-10 of 10 rows | 1-1 of 5 columns

```
[1] "6"
```

GO:0050853
GO:0050851
GO:0016064
GO:0019724
GO:0002429
GO:0002768
GO:0002250
GO:0002449
GO:0002460
GO:0002757

1-10 of 10 rows | 1-1 of 5 columns

Hide

```
# Report total run time Seurat pipeline
toc(quiet = FALSE)
```

```
Total time Seurat pipeline: 1474.222 sec elapsed
```