

Supplementary material 2 GEO: GSE280314, GSE280318

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1 Packages and data sets

1.1 Loading the required packages

```
pacman::p_load(spatstat,igraph,combinat,parallel)
source("functions_srt.R")
```

1.2 Creating the point pattern

1.2.1 Reading transcription event coordinates

The analysis is performed on different subimages delineated by an expert. Specifically, we focused on the same expert-defined tumor microenvironment regions previously analyzed in Oliveira et al. (2025) in their Figure 7, where Xenium in situ profiling was used to confirm the existence and spatial localization of macrophage subtypes and clonally expanded T cells. Images come from three patients (1, 2, and 5): areas c and e for patient 1, areas d and f for patient 2, and area h for patient 5.

The first step is to convert the transcription event files generated as described in Supplementary Material 1 into local spatial point patterns handled by R (format .rda).

```
PrependPath="/home/RAID5/SP_TRANS/FINAL_BMC_AREAS"
PrependPath=paste0(PrependPath,"/P")
for(i in c(1,2,5)){
  switch(as.character(i),
    "1" = {areasn = c("c","e")},
    "2" = {areasn = c("d","f")},
    "5" = {areasn = c("h")}
  )
  for(j in areasn){
    cat("Case ",i," Area ",j,"\n")
    dirbase = paste0(PrependPath,i,"/C",i,"_Area",j,"/")
    x = do.ppp(dirbase=dirbase,case=i,ff=1,min.n = 10)
    save(x,file=paste0("./data/local/x_l_case_",i,"_area_",j,"_ff_",1,
      ".rda"))
  }
}
```

```
Case 1 Area c
Case 1 Area e
Case 2 Area d
Case 2 Area f
Case 5 Area h
```

2 Statistical Analysis

2.1 Data-driven detection of atypical spatial interactions

1. For each pair of genes (i, j) , three interaction statistics are estimated: the cross-type K -function $(K_{ij}(r))$, the cross-type L -function $(L_{ij}(r))$, and the cross-type pair correlation function $(g_{ij}(r))$.
2. For each interaction statistic, a comparison envelope for the pair (i, j) is constructed from the corresponding functions of all other gene pairs, excluding (i, j) itself.
3. If the observed statistic for the pair (i, j) partially falls outside the comparison envelope, the interaction is *flagged as atypical*. Values above the envelope indicate spatial attraction or aggregation, values below indicate spatial repulsion or inhibition, and values within the envelope suggest no meaningful departure from the typical patterns observed across the system.
4. These atypical interactions are then used to construct gene–gene graphs, in which the width of each edge is set proportional to the number of distance intervals for which the corresponding interaction lies outside its comparison envelope.
5. The spatial distances at which the observed interaction statistics fall outside the envelopes are also identified.

2.2 Estimating cross functions

Estimates of the cross-type summary functions are obtained from the observed point patterns using the `Kcross`, `Lcross`, and `pcfcross` functions from the R package `spatstat` (Baddeley and Turner (2005)), and the results are saved.

```
detectCores()
```

```
[1] 128
```

```
rvalues = seq(0,152,2)
ff = 1
for(i in c(1,2,5)){
  switch(as.character(i),
    "1" = {areasn = c("c","e")},
    "2" = {areasn = c("d","f")},
    "5" = {areasn = c("h")}
  )
  for(j in areasn){
```

```

cat("Case ",i," Area ",j,"\n")
file_x = paste0("./data/local/x_l_case_",i,"_area_",j,"_ff_",1,".rda")
load(file_x)
sel = which(!is.na(x))
pairs1 = t(combn(sel,2))
x1 = x[sel]
X = do.call(superimpose, x1)
pairs11 = t(combn(length(sel),2))
kc.l = mclapply(1:nrow(pairs11),function(k){
  ## cat("k ",k,"\n")
  Kcross(X,i=levels(marks(X))[pairs11[k,1]],
          j=levels(marks(X))[pairs11[k,2]],r=rvalues,
          correction="border")
})
Lc.l = mclapply(1:nrow(pairs11),function(k){
  ## cat("k ",k,"\n")
  Lcross(X,i=levels(marks(X))[pairs11[k,1]],
          j=levels(marks(X))[pairs11[k,2]],r=rvalues,
          correction="border")
})
pcf.l = mclapply(1:nrow(pairs11),function(k){
  ## cat("k ",k,"\n")
  pcfcross(X,i=levels(marks(X))[pairs11[k,1]],
            j=levels(marks(X))[pairs11[k,2]],r=rvalues,
            correction="iso")
})
kc = matrix(unlist(lapply(kc.l,function(x) x$border)),
            ncol=length(rvalues),byrow=TRUE)
Lc = matrix(unlist(lapply(Lc.l,function(x) x$border)),
            ncol=length(rvalues),byrow=TRUE)
pcf = matrix(unlist(lapply(pcf.l,function(x) x$iso)),
            ncol=length(rvalues),byrow=TRUE)
save(kc,file=paste0("./results/local/kc_l_",i,"_ff_",ff,"_area_",j,
                    ".rda"))
save(Lc,file=paste0("./results/local/Lc_l_",i,"_ff_",ff,"_area_",j,
                    ".rda"))
save(pcf,file=paste0("./results/local/pcf_l_",i,"_ff_",ff,"_area_",j,
                    ".rda"))
}
}

```

Case 1 Area c

Case 1 Area e

Case 2 Area d

Case 2 Area f

Case 5 Area h

2.3 Finding atypical spatial interactions

For each interaction statistic, a reference envelope for the pair (i, j) is generated using the corresponding summary functions from all other gene pairs, excluding the pair (i, j) .

If the observed statistic for (i, j) extends partially beyond this reference envelope, the interaction is marked as atypical.

Additionally, we record on which side of the envelope the statistic exits (above or below), as this directionality provides further information about the nature of the deviation.

```
ff = 1
for(desc in c("kc","Lc","pcf")){
  for(i in c(1,2,5)){
    switch(as.character(i),
      "1" = {areasn = c("c","e")},
      "2" = {areasn = c("d","f")},
      "5" = {areasn = c("h")}
    )
    for(j in areasn){
      cat("Case ",i," Area ",j,"\n")
      file_desc = paste0("./results/local/",desc,"_l_",i,"_ff_",ff,"_area_",j,".rda")
      load(file_desc)
      switch(desc,
        "kc" = {sig = oneRow(x=kc,method="all")},
        "Lc" = {sig = oneRow(x=Lc,method="all")},
        "pcf" = {sig = oneRow(x=pcf,method="all")}
      )
      file_sig = paste0("./results/local/sig_",desc,"_l_",i,"_ff_",ff,"_area_",j,".rda")
      save(sig,file=file_sig)
    }
  }
}
```

Case 1 Area c

Case 1 Area e

Case 2 Area d

Case 2 Area f

Case 5 Area h

Case 1 Area c

Case 1 Area e

Case 2 Area d

Case 2 Area f

Case 5 Area h

Case 1 Area c

Case 1 Area e

Case 2 Area d

Case 2 Area f

Case 5 Area h

```
## side
```

```
ff = 1
```

```
for(desc in c("kc","Lc","pcf")){
```

```
  for(i in c(1,2,5)){
```

```
    switch(as.character(i),
```

```
      "1" = {areasn = c("c","e")},
```

```
      "2" = {areasn = c("d","f")},
```

```
      "5" = {areasn = c("h")}
```

```
    )
```

```
    for(j in areasn){
```

```
      cat("Case ",i," fraction ", ff," area ",j,"\n")
```

```
      file_desc = paste0("./results/local/",desc,"_l_",i,"_ff_",ff,"_area_",j,".rda")
```

```
      file_sig = paste0("./results/local/sig_",desc,"_l_",i,"_ff_",ff,"_area_",j,".rda")
```

```
      load(file_desc)
```

```
      load(file_sig)
```

```
      switch(desc,
```

```
        "kc" = {side_out = oneRowSide(x=kc,sig=sig)},
```

```
        "Lc" = {side_out = oneRowSide(x=Lc,sig=sig)},
```

```

        "pcf" = {side_out = oneRowSide(x=pcf,sig=sig)}
    )
    file_sig_side = paste0("./results/local/sig_side_",desc,"_l_",i,"_ff_",ff,"_area_",
    side=side_out$side
    save(side,file=file_sig_side)
}
}
}

```

Case 1 fraction 1 area c

Case 1 fraction 1 area e

Case 2 fraction 1 area d

Case 2 fraction 1 area f

Case 5 fraction 1 area h

Case 1 fraction 1 area c

Case 1 fraction 1 area e

Case 2 fraction 1 area d

Case 2 fraction 1 area f

Case 5 fraction 1 area h

Case 1 fraction 1 area c

Case 1 fraction 1 area e

Case 2 fraction 1 area d

Case 2 fraction 1 area f

Case 5 fraction 1 area h

2.4 Construction of graphs from genes with atypical interactions

2.4.1 Mapping ENSEMBL IDs to gene symbols

First, we establish the correspondence between Ensembl identifiers and gene symbols to be used in the graphs.

```

genes = read.csv("GSE280314_gene_panel.csv",header=TRUE,sep="\t")
ensembl = genes$id[1:100]
hgnc_symbol = genes$name[1:100]
save(ensembl,hgnc_symbol,file="annotation.rda")

```

2.4.2 Graphs with local interactions

Next, we generate the graphs for local point patterns.

```

ff = 1
for(desc in c("kc","Lc")){
  for(i in c(1,2,5)){
    switch(as.character(i),
      "1" = {areasn = c("c","e")},
      "2" = {areasn = c("d","f")},
      "5" = {areasn = c("h")}
    )
    for(j in areasn){
      cat("desc ",desc,"Case ",i," Area ",j,"\n")
      file_x = paste0("./data/local/x_l_case_",i,"_area_",j,"_ff_",1,".rda")
      file_sig = paste0("./results/local/sig_",desc,"_l_",i,"_ff_",ff,"_area_",j,
        ".rda")
      file_sig_side = paste0("./results/local/sig_side_",desc,"_l_",i,"_ff_",ff,
        "_area_",j,".rda")
      file_annotation = "annotation.rda"
      g = do_graph(file_x,file_sig,file_sig_side,file_annotation)
      file_plot=paste0("./figures/local/graph_",desc,"_l_",i,"_ff_",ff,"_area_",j,
        ".png")
      png(file_plot)
      plot(g)
      dev.off()
      file_graph = paste0("./figures/local/graph_",desc,"_l_",i,"_ff_",ff,"_area_",j,
        ".graph")
      save(g,file=file_graph)
      metag = data.frame(V1 = E(g)$label1,V2 = E(g)$label2,width = E(g)$width,
        out = factor(E(g)$out,levels=1:2,labels=c("low","up")))
      file_metag = paste0("./figures/local/metagraph_",desc,"_l_",i,"_ff_",ff,"_area_",
        ".csv")
      write.csv(metag,file=file_metag)
    }
  }
}

```

```
}  
}
```

```
desc kc Case 1 Area c  
  
desc kc Case 1 Area e  
  
desc kc Case 2 Area d  
  
desc kc Case 2 Area f  
  
desc kc Case 5 Area h  
  
desc Lc Case 1 Area c  
  
desc Lc Case 1 Area e  
  
desc Lc Case 2 Area d  
  
desc Lc Case 2 Area f  
  
desc Lc Case 5 Area h
```

2.5 Envelopes

2.5.1 Intervals where the descriptive is out the envelopes

```
ff = 1  
file_annotation = "annotation.rda"  
load(file_annotation)  
df_all = NULL  
for(desc in c("kc","Lc")){  
  for(i in c(1,2,5)){  
    switch(as.character(i),  
          "1" = {areasn = c("c","e")},  
          "2" = {areasn = c("d","f")},  
          "5" = {areasn = c("h")})
```

```

    )
  for(j in areasn){
    cat("desc ",desc,"Case ",i," Area ",j,"\n")
    file_x = paste0("./data/local/x_l_case_",i,"_area_",j,"_ff_",1,".rda")
    load(file_x)
    sel = which(!is.na(x))
    pairs1 = t(combn(sel,2))
    file_sig = paste0("./results/local/sig_",desc,"_l_",i,"_ff_",ff,"_area_",j,
                      ".rda")

    load(file_sig)
    pairs1 = pairs1[sig,]
    sel1 = sort(unique(c(pairs1[,1],pairs1[,2])))
    file_sig_side = paste0("./results/local/sig_side_",desc,"_l_",i,"_ff_",ff,
                           "_area_",j,".rda")

    load(file_sig_side)
    file_range =paste0("./results/local/ranges_",desc,"_l_",i,"_ff_",ff,"_area_",j,
                       ".csv")

    df = data.frame(desc= rep(desc,nrow(pairs1)),
                    case = rep(i,nrow(pairs1)),
                    window = rep(j,nrow(pairs1)),
                    gene1 = hgnc_symbol[pairs1[,1]],
                    gene2 = hgnc_symbol[pairs1[,2]],
                    lower = apply(side,1,function(u) min(which(u !=0))),
                    upper = apply(side,1,function(u) max(which(u !=0))))

    df_all = rbind(df_all,df)
    save(df,file = file_range)
  }
}

```

```

desc  kc Case  1  Area  c
desc  kc Case  1  Area  e
desc  kc Case  2  Area  d
desc  kc Case  2  Area  f
desc  kc Case  5  Area  h
desc  Lc Case  1  Area  c
desc  Lc Case  1  Area  e
desc  Lc Case  2  Area  d
desc  Lc Case  2  Area  f
desc  Lc Case  5  Area  h

```

```
save(df_all,file = "./results/local/df_all.rda")
```

Baddeley, A., and R. Turner. 2005. "Spatstat: An R Package for Analyzing Spatial Point Patterns." *Journal of Statistical Software* 12: 6. <https://doi.org/10.18637/jss.v012.i06>.

Oliveira, Michelli Faria de, Juan Pablo Romero, Meii Chung, et al. 2025. "High-Definition Spatial Transcriptomic Profiling of Immune Cell Populations in Colorectal Cancer." *Nature Genetics* 57 (June): 6. <https://doi.org/10.1038/s41588-025-02193-3>.