Vignette

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This package was built to facilitate the analysis of longitudinal metabolomics data. Common tools mostly only allow the comparison between two time points or experimental conditions and are using frequentist statistical methods.

Here we want to show a complete workflow to analyze concentration tables. As example we have a data set of an irradiated cancer cell line that was observed over four time points after irradiation with different doses. For each timepoint and radiation dose it contains three replicates of "cpc-values". Cpc refers here to the metabolite concentration per cell.

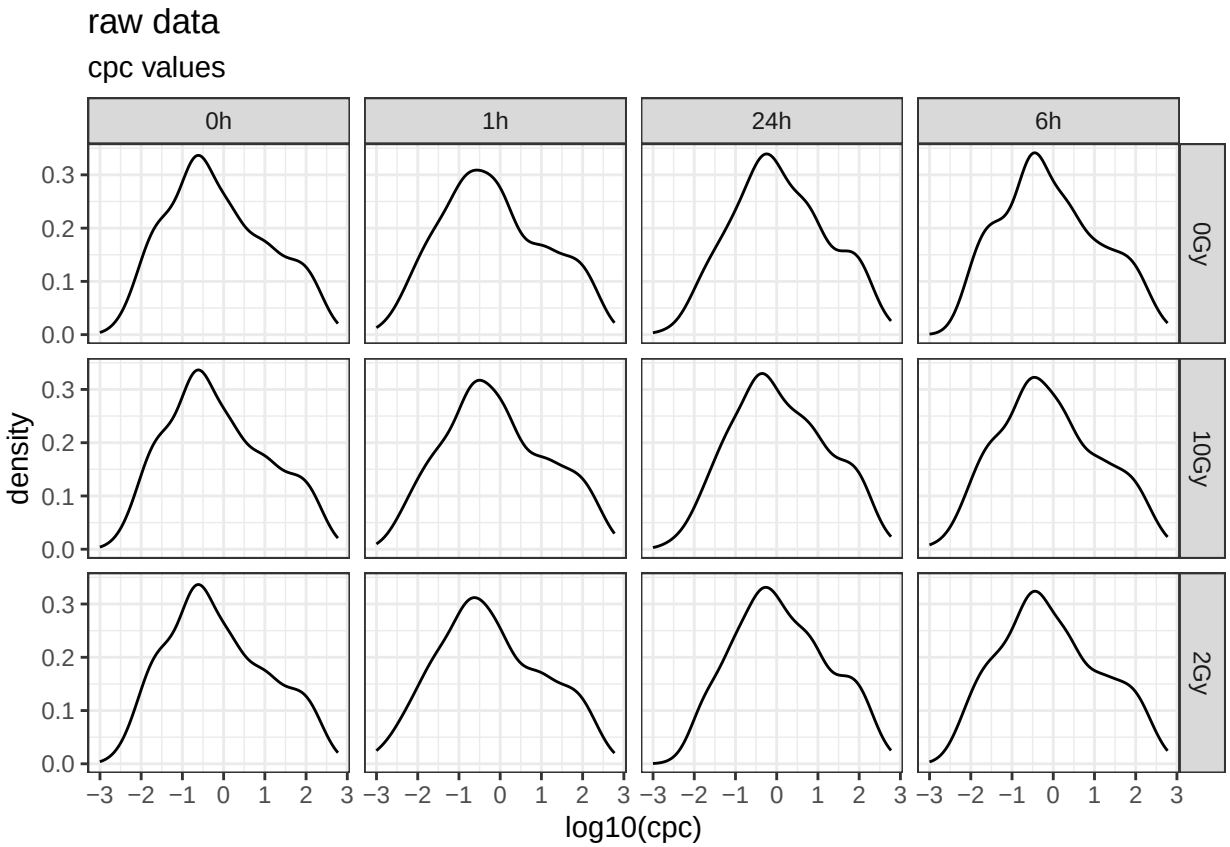
setup: load required packages

```
library(MetaboDynamics)
library(ggplot2)
library(dplyr)
library(tidyr)
```

load data and plot data overview

```
data("intra")
```

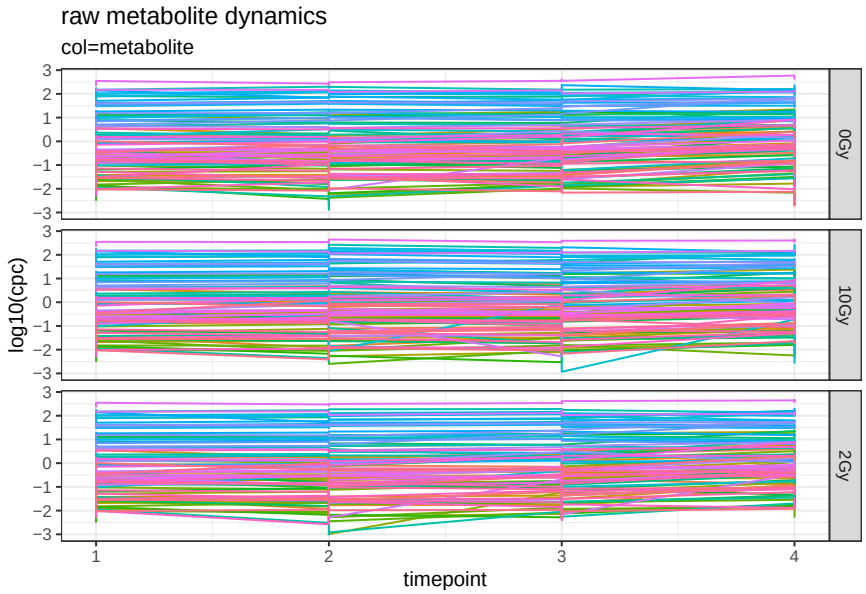
```
# plot log-transformed data
ggplot(intra, aes(x = log10(cpc))) +
  geom_density() +
  theme_bw() +
  facet_grid(cols = vars(time), rows = vars(dose)) +
  ggtitle("raw data", "cpc values")
```



In the first step in this workflow we estimate the dynamics of every single metabolite at every experimental condition (here: radiation dose). As metabolomics data is often noisy and we generally have few replicates due to high costs a robust method is needed for the estimation of mean concentrations at every time point. For this employ a Bayesian hierarchical model that assumes normal distributions of log-transformed metabolite concentrations. The next plot shows the raw dynamics of single metabolites

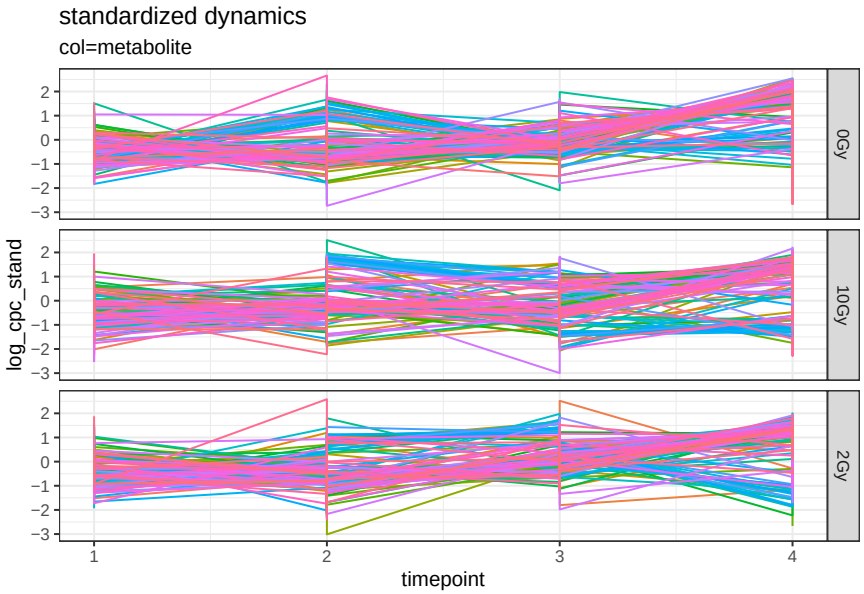
get time as numeric variable

```
ggplot(intra) +
  geom_line(aes(x = as.numeric(as.factor(time)), y = log10(cpc),
                col = metabolite, group=metabolite)) +
  theme_bw() +
  xlab("timepoint") +
  theme(legend.position = "none") +
  facet_grid(rows = vars(dose)) +
  ggtitle("raw metabolite dynamics", "col=metabolite")
```



We define dynamics as deviations at the observed time points from the metabolite's mean concentration over time. As the raw concentrations of metabolites can differ by magnitudes from each other, and we want to be able to compare dynamics of metabolites with each other, we standardize each metabolite at each radiation dose to a mean of zero and a standard deviation of one.

```
intra <- intra %>%
  group_by(dose, metabolite) %>%
  mutate(log_cpc_stand = ((log_cpc - mean(log_cpc)) / sd(log_cpc)))
ggplot(intra) +
  geom_line(aes(
    x = as.numeric(as.factor(time)),
    y = log_cpc_stand, col = metabolite
  )) +
  theme_bw() +
  xlab("timepoint") +
  theme(legend.position = "none") +
  facet_grid(rows = vars(dose)) +
  ggtitle("standardized dynamics", "col=metabolite")
```



Now we can finally model the dynamics. The code below shows how to fit the model and how to extract the diagnostic criteria from the model fits.

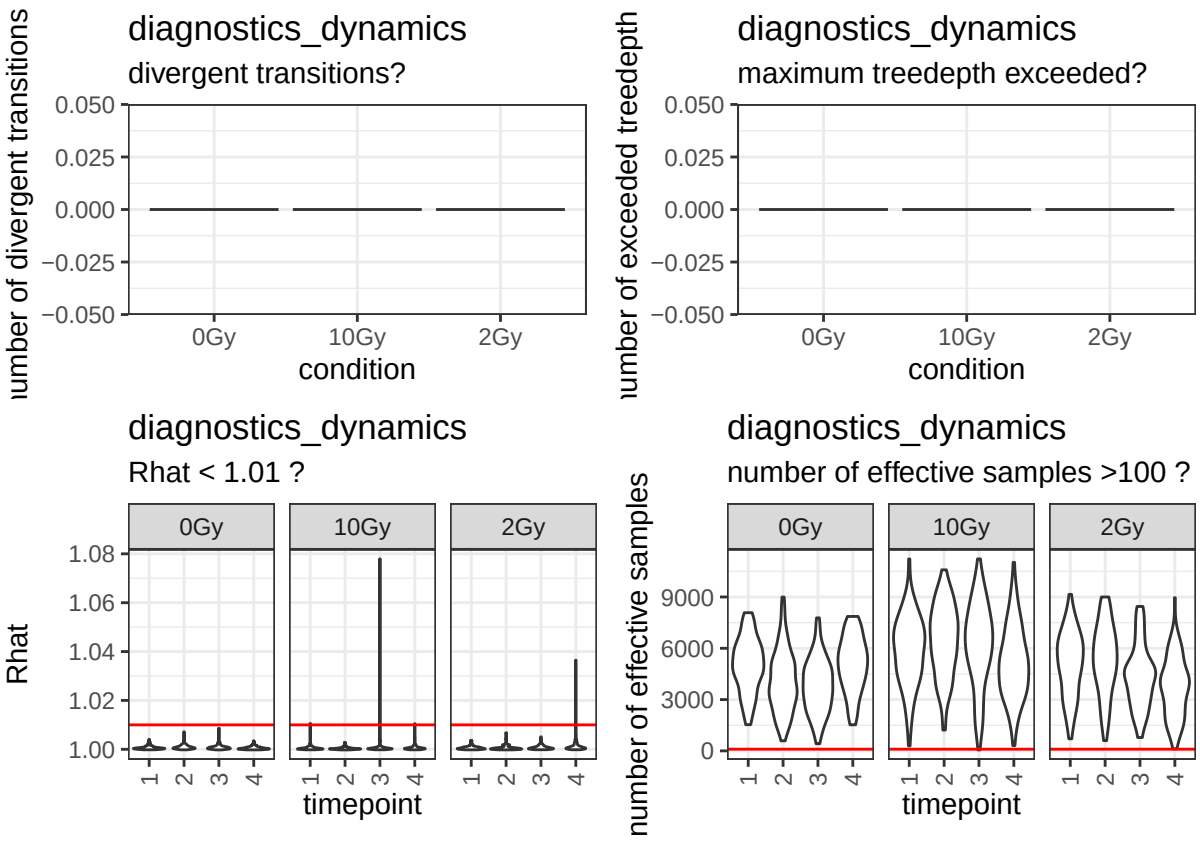
Model dynamics

```
# # fit model
# fits_dynamics <- fit_dynamics_model(
#   data = intra, cpc = "log_cpc_stand",
#   condition = "dose", max_treedepth = 14,
#   adapt_delta = 0.999, iter = 4000, cores = 7)

# # extract diagnostics
# diagnostics_dynamics <- extract_diagnostics_dynamics(
#   data = intra, iter = 4000,
#   fits = fits_dynamics
# )

# diagnostics_dynamics[["plot_divergences"]]
# diagnostics_dynamics[["plot_treedepth_error"]]
# diagnostics_dynamics[["plot_rhat"]]
# diagnostics_dynamics[["plot_neff"]]

# # PPCs can be accessed with
# diagnostic_estimates[["plot_PPC_0Gy"]]
# diagnostic_estimates[["plot_PPC_2Gy"]]
# diagnostic_estimates[["plot_PPC_10Gy"]]
```



For our experimental data set this might take up to 10 minutes per radiation dose depending on how many cores are used.

This returns a list of model fits that are named by the experimental condition ("0Gy","2Gy","10Gy"). With extract_diagnostics_dynamics() we can extract all the diagnostic criteria of Bayesian models (rhat,neff,divergences,max_treedepth) and visualize them.

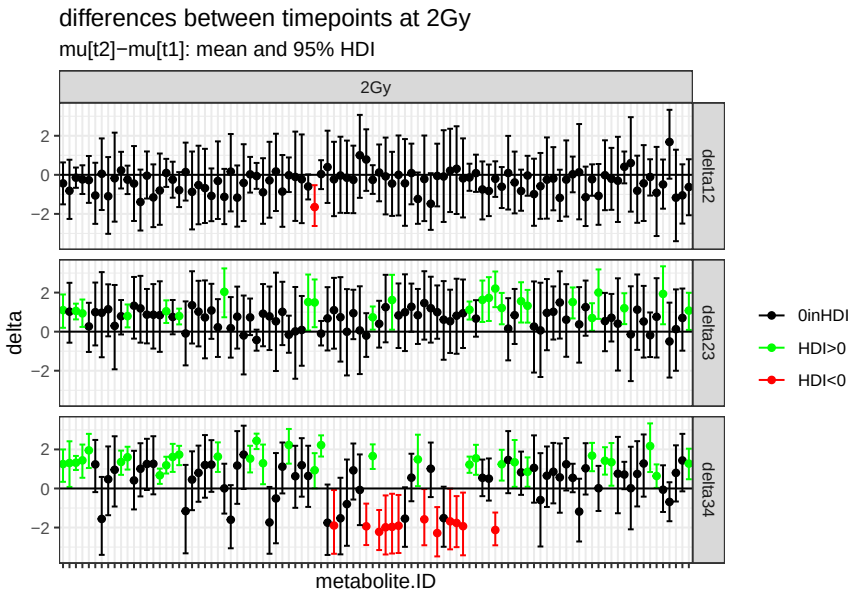
After checking the diagnostic criteria and the PPC we can extract the estimates:

```
# #extract estimates
# estimates_dynamics <- extract_estimates_dynamics(
#   data = intra, fits = fits_dynamics,
#   iter = 4000
# )
```

We get two major outputs: 1) the estimation of concentration differences between two subsequent time points of each metabolite at each experimental condition 2) the dynamic profiles of each metabolites at each experimental condition

1) differences between two timepoints

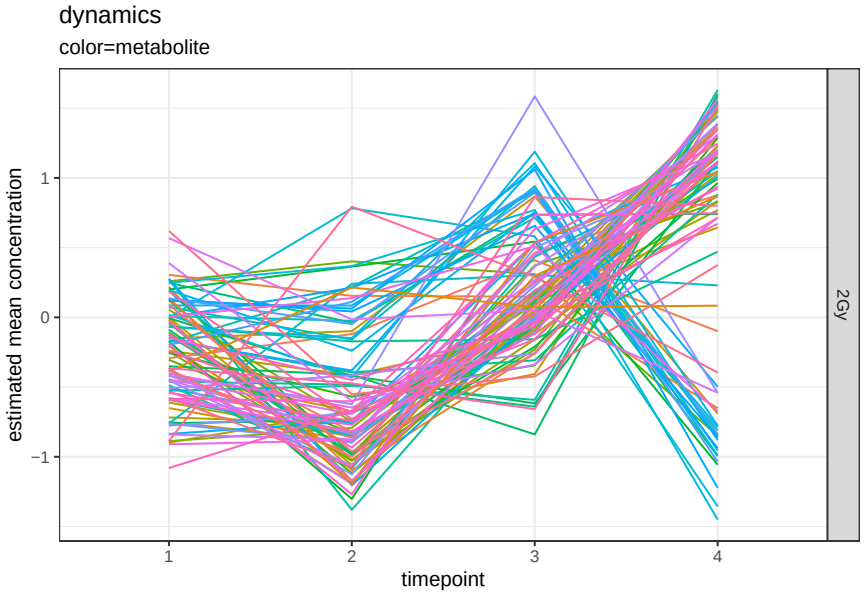
```
# 1) the differences between two timepoints
# set numer of timepoints
#estimates_dynamics[["plot_timepoint_differences"]]
```



If the 95% highest density interval of the posterior does not include zero we can confidently state that there is a difference in mean concentrations between two time points. If the 95% HDI is smaller than zero we have a decrease in metabolite concentrations between the two time points, if it is greater than zero we have an increase in concentrations between time points.

2) dynamic profiles

```
# 2) dynamic profiles
#estimates_dynamics[["plot_dynamics"]]
```



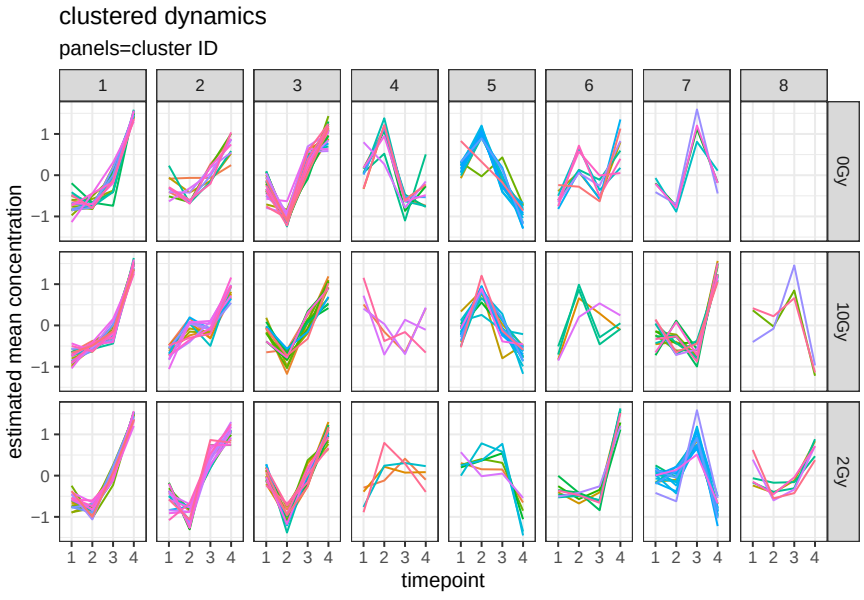
So we now have dynamic profiles of the metabolites at each radiation dose. What do we do with this? We

could cluster these dynamics vectors, that we obtained to investigate if we find groups of metabolites that have similar dynamics.

cluster dynamics

For the sake of demonstration we only show the results of the clustering analysis here. In practice the optimal number of clusters should be determined by clustering criteria such as Gap statistics and average silhouette.

```
temp <- cluster
temp <- temp %>% pivot_longer(
  cols = c(mu1_mean, mu2_mean, mu3_mean, mu4_mean),
  names_to = "timepoint", values_to = "mu_mean"
)
ggplot(temp, aes(
  x = as.factor(as.numeric(as.factor(timepoint))),
  y = mu_mean, group = metabolite, col=metabolite
)) +
  geom_line() +
  xlab("timepoint") +
  ylab("estimated mean concentration") +
  theme_bw() +
  theme(legend.position = "none") +
  facet_grid(rows = vars(condition), cols = vars(cluster)) +
  ggtitle("clustered dynamics", "panels=cluster ID")
```



As we can see metabolites show different dynamics in different radiation doses. Can we quantify the biological function of these dynamic clusters?

Over-representation analysis of functional modules in dynamic clusters

To quantify the possible biological function of these dynamic clusters we retrieved from the KEGG-database the following information (with package KEGGREST): 1) to which functional modules our experimental metabolites are annotated and 2) which metabolites are annotated to functional modules in general

The functional modules of the KEGG-database are organised in three hierarchies: upper, middle and lower. Here we will do functional analysis on the middle hierarchy. To facilitate analysis the data frames "metabolite_modules" which holds the information about experimental metabolites and "modules_compounds" which holds the information about which metabolites are in general annotated to functional modules. We load both data sets and can inspect the documentation.

```
data("metabolite_modules")
#help("metabolite_modules")
head(metabolite_modules)
```

```
## # A tibble: 6 x 8
##    ...1 metabolite    KEGG  module_id module_name upper_hierachy middle_hierachy
##   <dbl> <chr>         <chr> <chr>     <chr>       <chr>          <chr>
## 1     1 1-Aminocyclo~ C012~ M00368    Ethylene b~ Pathway modul~ Amino acid met~
## 2     2 2-Aminomucon~ C022~ M00038    Tryptophan~ Pathway modul~ Amino acid met~
## 3     3 2-Phosphogly~ C006~ M00001    Glycolysis~ Pathway modul~ Carbohydrate m~
## 4     4 2-Phosphogly~ C006~ M00002    Glycolysis~ Pathway modul~ Carbohydrate m~
## 5     5 2-Phosphogly~ C006~ M00003    Gluconeoge~ Pathway modul~ Carbohydrate m~
## 6     6 2-Phosphogly~ C006~ M00346    Formaldehy~ Pathway modul~ Energy metabol~
## # i 1 more variable: lower_hierachy <chr>
```

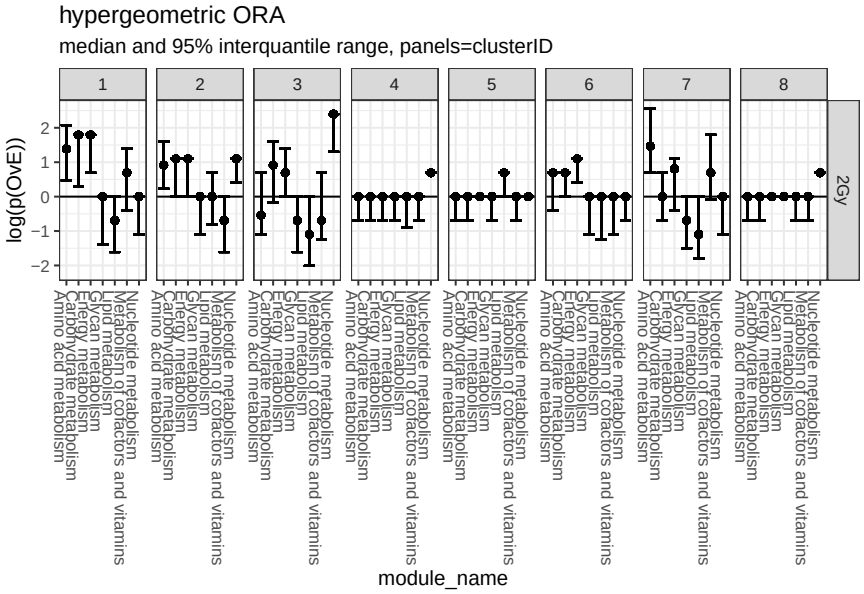
```
data("modules_compounds")
#help("modules_compounds")
head(modules_compounds)
```

```
## # A tibble: 6 x 6
##    ...1 module_id kegg_id upper_hierachy  middle_hierachy         lower_hierachy
##   <dbl> <chr>     <chr>   <chr>           <chr>                   <chr>
## 1     2 M00001    C00267  Pathway modules Carbohydrate metabolism Central carbo~
## 2     3 M00001    C00668  Pathway modules Carbohydrate metabolism Central carbo~
## 3     4 M00001    C05345  Pathway modules Carbohydrate metabolism Central carbo~
## 4     5 M00001    C05378  Pathway modules Carbohydrate metabolism Central carbo~
## 5     6 M00001    C00111  Pathway modules Carbohydrate metabolism Central carbo~
## 6     7 M00001    C00118  Pathway modules Carbohydrate metabolism Central carbo~
```

For the functional analysis we employ a hypergeometric test. We consider a functional module as over-expressed in a cluster if the probability of the distribution of OvEs is greater than 1. OvE refers to the ratio of observed metabolites in a cluster being mapped to a functional module over the number of expected metabolites in a cluster being mapped to a module under the assumption of a hypergeometric distribution (=drawing without replacement).

```
ORA <- ORA_hypergeometric(background = modules_compounds,
                          annotations = metabolite_modules,
                          clusters = cluster, tested_column = "middle_hierachy")
a_clusters <- ORA[["ORA"]]
```

```
ggplot(a_clusters[a_clusters$condition=="2Gy",],
       aes(y=log(OvE_gen),x=module_name))+
      geom_point(aes(y=log(OvE_gen_median)))+
      geom_errorbar(aes(ymin=log(OvE_gen_lower),ymax=log(OvE_gen_higher)))+
      geom_hline(yintercept=0)+
      theme_bw()+
      ylab("log(p(OvE))")+
      theme(axis.text.x=element_text(angle = -90, hjust = 0))+
      facet_grid(cols=vars(cluster),rows=vars(condition))+
      ggtitle("hypergeometric ORA",
              "median and 95% interquantile range, panels=clusterID")
```



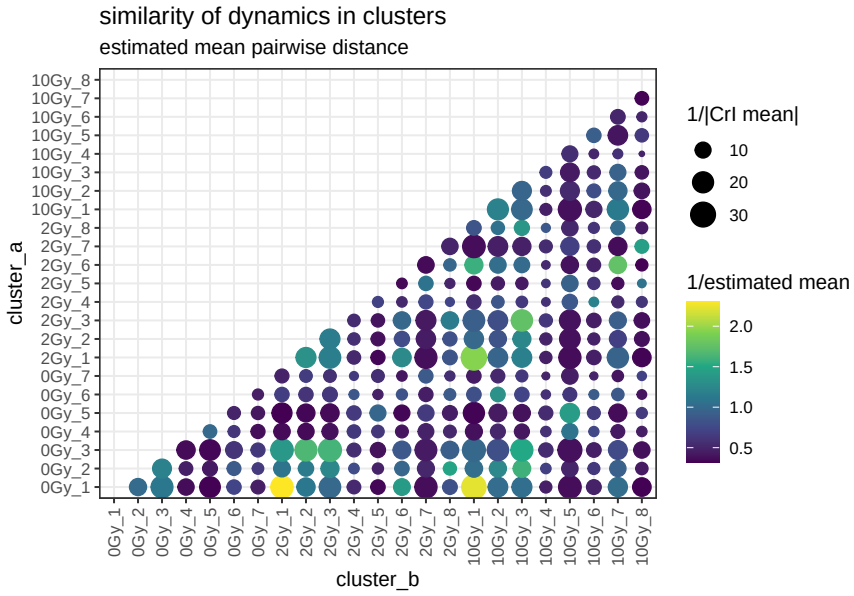
Great, we can now see which functional module is over- or under-represented in which dynamic cluster!

Comparison of clusters of different experimental conditions

dynamics

We can not only do over-representation analysis of KEGG-functional modules but also compare dynamic clusters across different experimental conditions. For this we employ a Bayesian model that estimates the mean difference as well as the standard deviation of differences between dynamic clusters.

```
comparison_dynamics <- compare_dynamics(clusters = cluster,
                                        dynamics = c("mu1_mean", "mu2_mean",
                                                     "mu3_mean", "mu4_mean"))
comparison_dynamics[["plot_dynamic_comparison"]]
```

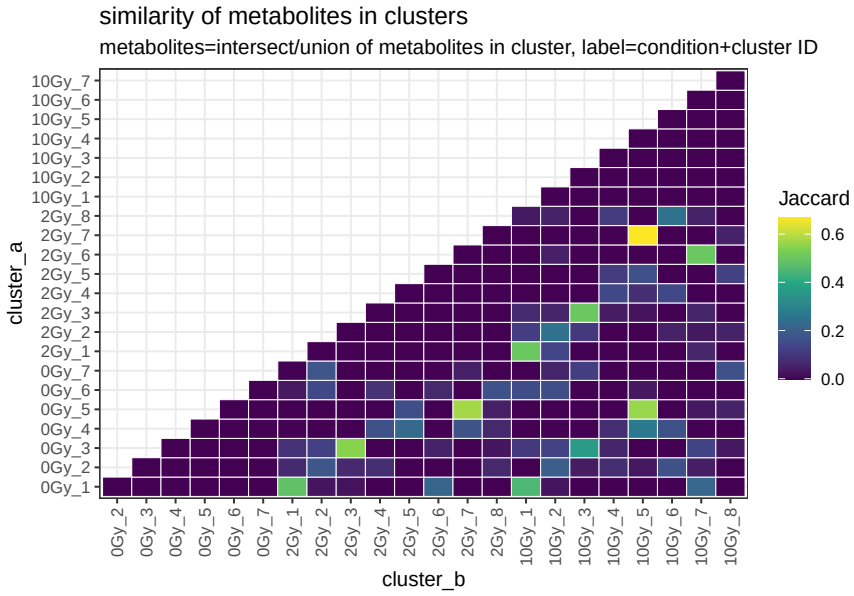


The bigger and brighter a point is the smaller is the mean distance between dynamic clusters and the smaller is the standard deviation. That means big bright points indicate high dynamic similarity which small spread.

metabolites

For the comparison of metabolites between different dynamic clusters we employ the Jaccard Index.

```
comparison_metabolites <- compare_metabolites(clusters=cluster)
comparison_metabolites[["plot_metabolite_comparison"]]
```

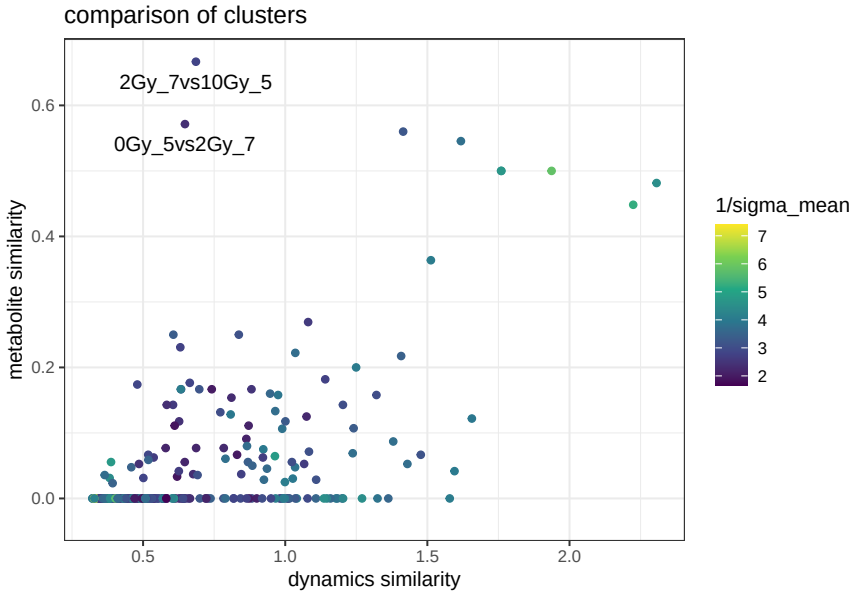


We have two clusters that are very similar in their metabolite composition. If we compare that to the dynamic profiles and ORA analysis we see that similar functional modules are over-expressed as expected BUT the dynamics differ between the two radiation doses.

Can we facilitate visualization?

combine both

```
dynamics <- comparison_dynamics[["estimates"]]
metabolites <- comparison_metabolites[["Jaccard"]]
temp <- left_join(dynamics,metabolites,by=c("cluster_a","cluster_b"))
ggplot(temp,aes(x=1/mu_mean,y=Jaccard))+
  geom_point(aes(col=1/sigma_mean))+
  theme_bw()+
  geom_text(aes(label=ifelse(Jaccard>0.4&1/mu_mean<1.0,comparison,NA)),
            nudge_y=-0.03)+
  scale_color_viridis_c()+
  xlab("dynamics similarity")+
  ylab("metabolite similarity")+
  ggtitle("comparison of clusters")
```



We can find two cluster pairs that are pretty similar in regards to their composing metabolites but dissimilar in regards to their dynamics. Their ORA profiles are quiet similar as expected from the similar metabolite compositions.