

A Bioconductor workflow for the Bayesian Analysis of Spatial proteomics

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1 Introduction

Quantifying uncertainty in the spatial subcellular distribution of proteins allows for novel insight into protein function (O. M. Crook et al. 2018). Many proteins live in a single location within the cell, however there are those that reside in multiple locations and those that dynamically relocate (Thul et al. 2017). These phenomena lead to variability and uncertainty in robustly assigning proteins a unique localisation. Functional compartmentalisation of proteins allows the cell to control biomolecular pathways and biochemical process within the cell. Therefore, proteins with multiple localisation may have multiple functional roles (Jeffery 2009). Machine learning algorithms that fail to quantify uncertainty are unable to draw deeper insight into understanding cell biology from mass-spectrometry (MS) based spatial proteomics experiments. Henceforth, quantifying uncertainty allows us to make rigorous assessments of protein subcellular localisation and multi-localisation.

Bayesian approaches to machine learning and statistical analysis can provide more insight into the data, since uncertainty quantification arises as a consequence of a generative model for the data (A. Gelman et al. 1995). In a Bayesian framework, a model with parameters for the data is proposed, along with a statement about our prior beliefs of the model parameters. Bayes’ theorem tells us how to update the prior distribution of the parameters to obtain the posterior distribution of the parameters after observing the data. It is the posterior distribution which quantifies the uncertainty in the parameters and quantities of interest derived from the data. This contrasts from a maximum-likelihood approach where we obtain only a point estimate of the parameters.

Adopting a Bayesian framework for data analysis, though of much interest to experimentalists, can be challenging. Once we have obtained a probabilistic model, complex algorithms are used to obtain the posterior distribution upon observation of the data. These algorithms can have tuning parameters and many settings, hindering their practical use for those not versed in Bayesian methodology. Even once the algorithms have been correctly set-up, assessments of convergence and guidance on how to interpret the results are often sparse. This workflow presents a Bayesian analysis of spatial proteomics to elucidate the process to any practitioners. We hope that it goes beyond simply the methods, data structures and biology presented here, but provides a template for others to design tools using Bayesian methodology for the biological community.

Our model for the data is the t-augmented Gaussian mixture (TAGM) model proposed in (O. M. Crook et al. 2018). O. M. Crook et al. (2018) provide a detailed description of the model, rigorous comparisons and testing on many spatial proteomics datasets and a case study on a hyperLOPIT experiment on mouse pluripotent stem cells (Christoforou et al. 2016; Mulvey et al. 2017). Revisiting these details is not the purpose of this computational protocol, rather we present how to correctly use the software and provide step by step guidance for interpreting the results.

In brief, the TAGM model posits that each annotated sub-cellular niche can be described by a Gaussian distribution. Thus the full complement of proteins within the cell is captured as a mixture of Gaussians. The highly dynamic nature of the cell means that many proteins are not well captured by any of these multivariate Gaussian distributions, and thus the model also includes an outlier component, mathematically described as multivariate student’s t distribution. The heavy tails of the t distribution allow it to better capture dispersed proteins.

To perform inference in the TAGM model there are two approaches. The first, which we refer to as TAGM MAP, allows us to obtain *maximum a posteriori* estimates of posterior localisation probabilities; that is,

the modal posterior probability that a protein localises to that class. This approach uses the expectation-maximisation (EM) algorithm to perform inference (Dempster, Laird, and Rubin 1977). Whilst this is an interpretable summary of the TAGM model, it only provides point estimates. For a richer analysis, we present a Markov-chain Monte-Carlo (MCMC) method to perform fully Bayesian inference in our model, allowing us to obtain full posterior localisation distributions. This method is referred to as TAGM MCMC throughout the text.

This workflow begins with a brief review of some of the basic features of mass spectrometry-based spatial proteomics data, including the state-of-the-art computational infrastructure and bespoke software suite. We then present each method in turn, detailing how to obtain high quality results. We provide an extended discussion of the TAGM MCMC method to highlight some of the challenges when applying this method. This includes how to assess convergence of MCMC methods, as well as methods for manipulating the output. We then take the processed output and explain how to interpret the results, as well as providing some tools for visualisation. We conclude with some remarks and directions for the future.

2 Getting started and infrastructure

In this workflow, we are currently using version 1.23.2 of `pRoloc` (Gatto et al. 2014). The package `pRoloc` contains algorithms and methods for analysing spatial proteomics data, building on the `MSnSet` structure provided in `MSnbase`. The `pRolocdata` package provides many annotated datasets from a variety of species and experimental procedures. The following code chunks install and load the suite of packages required for the analysis.

```
if (!require("BiocManager"))
  install.packages("BiocManager")
BiocManager::install(c("pRoloc", "pRolocdata"))

library("pRoloc")

##
## This is pRoloc version 1.23.2
## Visit https://lgatto.github.io/pRoloc/ to get started.

library("pRolocdata")

##
## This is pRolocdata version 1.20.0.
## Use 'pRolocdata()' to list available data sets.
```

We assume that we have a MS-based spatial proteomics dataset contained in a `MSnSet` structure. For information on how to import data, perform basic data processing, quality control, supervised machine learning and transfer learning see following workflow (Breckels et al. 2016). We use a spatial proteomics dataset on mouse E14TG2a embryonic stem cells (Breckels et al. 2013). The LOPIT protocol (Dunkley et al. 2004; Dunkley et al. 2006) was used and normalised intensity of proteins from eight iTRAQ 8-plex labelled fraction are provided. The methods provided here are independent of labelling procedure, fractionation process or workflow. Examples of valid experimental protocols are LOPIT (Dunkley et al. 2004), hyperLOPIT (Christoforou et al. 2016; Mulvey et al. 2017), label-free methods such as PCP (Foster et al. 2006), and when fractionation is performed by differential centrifugation (Itzhak et al. 2016; Geladaki et al. 2018).

In the code chunk below, we load the aforementioned dataset. The printout demonstrates that this experiment quantified 2031 proteins over 8 fractions.

```
data("E14TG2aR") # load experimental data
E14TG2aR

## MSnSet (storageMode: lockedEnvironment)
```

```
## assayData: 2031 features, 8 samples
##   element names: exprs
## protocolData: none
## phenoData
##   sampleNames: n113 n114 ... n121 (8 total)
##   varLabels: Fraction.information
##   varMetadata: labelDescription
## featureData
##   featureNames: Q62261 Q9JHU4 ... Q9EQ93 (2031 total)
##   fvarLabels: Uniprot.ID UniprotName ... markers (8 total)
##   fvarMetadata: labelDescription
## experimentData: use 'experimentData(object)'
## Annotation:
## - - - Processing information - - -
## Loaded on Thu Jul 16 15:02:29 2015.
## Normalised to sum of intensities.
## Added markers from 'mrk' marker vector. Thu Jul 16 15:02:29 2015
## MSnbase version: 1.17.12
```

On figure 1 (left), we can visualise the mouse stem cell dataset use the `plot2D` function. We observe that some of the organelle classes overlap and this is a typical feature of biological datasets. Thus, it is vital to perform uncertainty quantification when analysing biological data.

```
plot2D(E14TG2aR)
addLegend(E14TG2aR, where = "topleft", cex = 0.6)
```

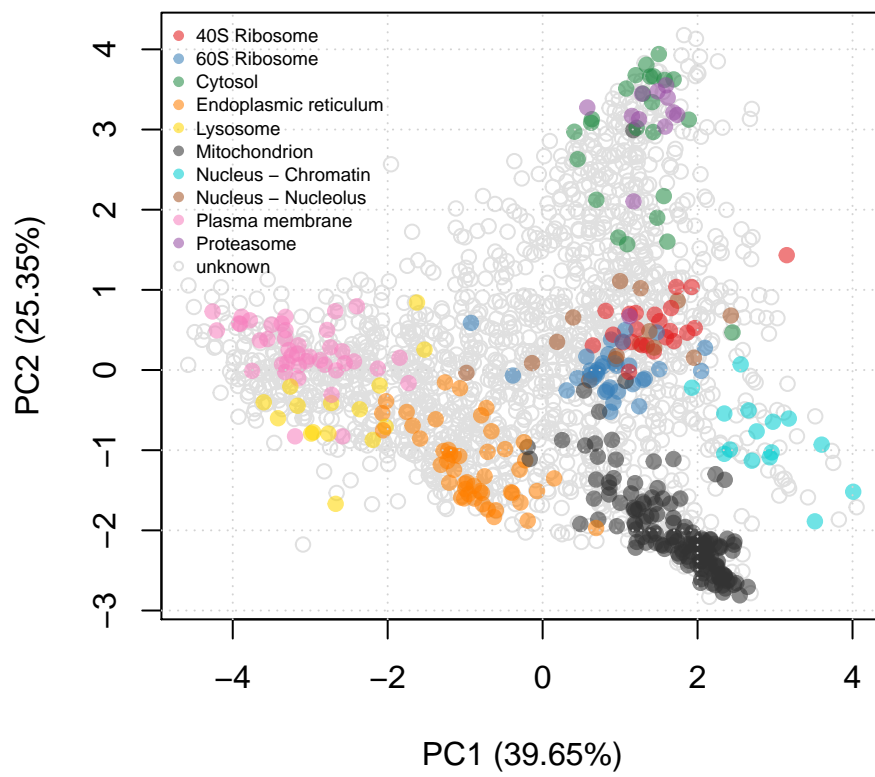


Figure 1: First two principal components of mouse stem cell data.

3 Methods: *TAGM MAP*

3.1 Introduction to TAGM MAP

We can perform *maximum a posteriori* (MAP) estimation to perform Bayesian inference in our model. The *maximum a posteriori* estimate equals the mode of the posterior distribution and can be used to provide a point estimate summary of the posterior localisation probabilities. It does not provide samples from the posterior distribution, however an extended version of the expectation-maximisation (EM) algorithm can be used in our case, allowing fast inference. The EM algorithm is an algorithm that iterates between an expectation step and a maximisation step. This allows us to find parameters which maximise the logarithm of the posterior, in the presence of latent (unobserved) variables. The EM algorithm is guaranteed to converge to a local mode. The code chunk below excutes the `tagmMapTrain` function for a default of 100 iterations. We use the default priors for simplicity and convenience, however they can be changed, which we explain in a later section. The output is an object of class `MAPPparams`, that captures the details of the TAGM MAP model.

```
set.seed(2)
mappars <- tagmMapTrain(E14TG2aR)

## co-linearity detected; a small multiple of
##           the identity was added to the covariance
mappars

## Object of class "MAPPparams"
## Method: MAP
```

Aside: co-linearity

The previous code chunk outputs a message concerning data co-linearity. This is because the covariance matrix of the data has become ill-conditioned and as a result the inversion of this matrix becomes unstable with floating point arithmetic. This can lead to the failure of standard matrix algorithms upon which our method depends. It is standard practice to add a small ridge; that is, a small multiple of the identity to stabilise this matrix. The printed message is a statement that this operation has been performed for these data.

3.2 Model visualisation

The results of the modelling can be visualised with the `plotEllipse` function on figure 2. The outer ellipse contains 99% of the total probability whilst the middle and inner ellipses contain 95% and 90% of the probability respectively. The centres of the clusters are represented by black circumpunct (circled dot). We can also plot the model in other principal components. The code chunk below plots the probability ellipses along the first and second, as well as the fourth prinipal component. The user can change the components visualised by altering the `dims` argument.

```
par(mfrow = c(1, 2))
plotEllipse(E14TG2aR, mappars)
plotEllipse(E14TG2aR, mappars, dims = c(1, 4))
```

3.3 The expectation-maximisation algorithm

The EM algorithm is iterative; that is, the algorithm iterates between an expectation step and a maximisation step until the value of the log-posterior does not change (Dempster, Laird, and Rubin 1977). This fact can be

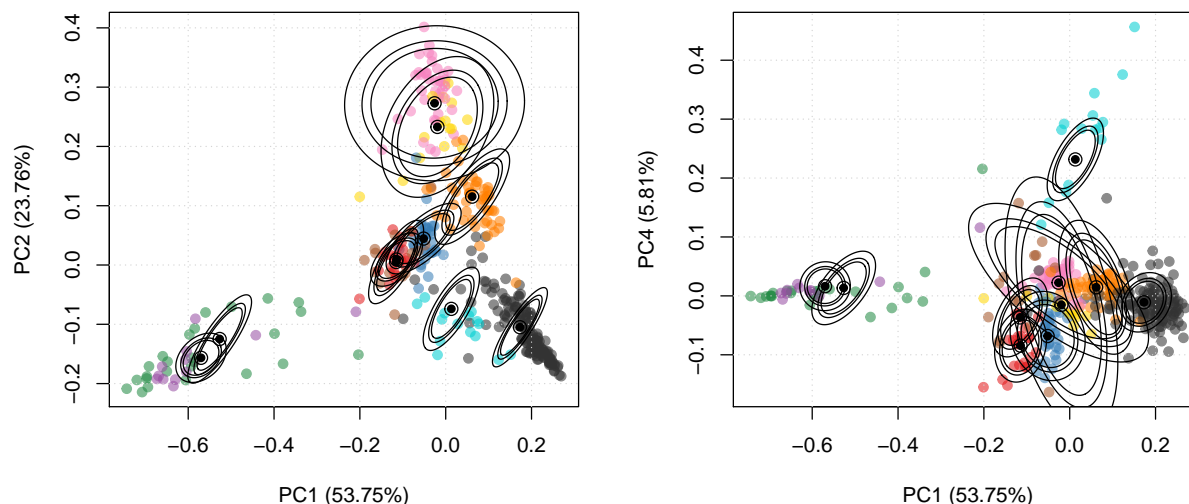


Figure 2: PCA plot with probability ellipses along PC 1 and 2 (left) and PC 1 and 4 (right)

used to assess the convergence of the EM algorithm. The value of the log-posterior at each iteration can be accessed with the `logPosteriors` function on the `MAPPparams` object. The code chunk below plots the log posterior at each iteration and we see on figure 3 the algorithm rapidly plateaus and so we have achieved convergence. If convergence has not been reached during this time, increase the number of iteration by changing the parameter `numIter` in the `tagmMapTrain` method. In practice, it is not unexpected to observe small fluctuations due to numerical errors and this should not concern users.

```
plot(logPosteriors(mappars), type = "b", col = "blue",
     cex = 0.3, ylab = "log-posterior", xlab = "iteration")
```

The code chunk below uses the `MAPPparams` object to classify the proteins of unknown localisation using `tagmPredict` function. This method appends new columns to the `fData` columns of the `MSnSet`.

```
E14TG2aR <- tagmPredict(E14TG2aR, mappars) # Predict protein localisation
```

The new feature variables that are generated are:

- `tagm.map.allocation`: the TAGM MAP predictions for the most probable protein sub-cellular allocation.

```
table(fData(E14TG2aR)$tagm.map.allocation)
```

```
##
##      40S Ribosome      60S Ribosome      Cytosol
##              34              85              328
## Endoplasmic reticulum      Lysosome      Mitochondrion
##              284              147              341
## Nucleus - Chromatin  Nucleus - Nucleolus  Plasma membrane
##              143              322              326
##      Proteasome
##              21
```

- `tagm.map.probability`: the posterior probability for the protein sub-cellular allocations.

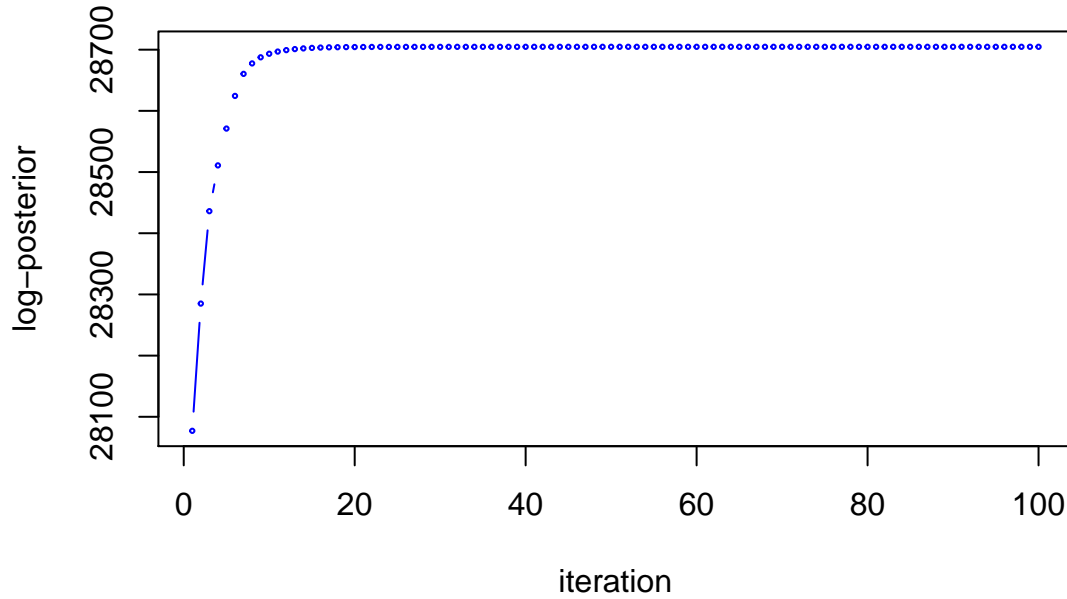


Figure 3: Log-posterior at each iteration of the EM algorithm demonstrating convergence.

```
summary(fData(E14TG2aR)$tagm.map.probability)
```

```
##      Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
## 0.3121  0.9693  0.9995  0.9465  1.0000  1.0000
```

- tagm.map.outlier: the posterior probability for that protein to belong to the outlier component rather than any annotated component.

```
summary(fData(E14TG2aR)$tagm.map.outlier)
```

```
##      Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
##      0      0      0      0      0      0
```

We can visualise the results by scaling the pointer according the posterior localisation probabilities. To do this we extract the MAP localisation probabilities from the feature columns of the the `MSnSet` and pass these to the `plot2D` function (figure 4).

```
ptsze <- fData(E14TG2aR)$tagm.map.probability # Scale pointer size
plot2D(E14TG2aR, fcol = "tagm.map.allocation", cex = ptsze)
addLegend(E14TG2aR, where = "topleft", cex = 0.6, fcol = "tagm.map.allocation")
```

The TAGM MAP method is easy to use and it is simple to check convergence, however it is limited in that it can only provide point estimates of the posterior localisation distributions. To obtain the full posterior distributions and therefore a rich analysis of the data, we resort to using Markov-Chain Monte-Carlo methods. In our particular case, we use a so-called collapsed Gibbs sampler (A. F. Smith and Roberts 1993).

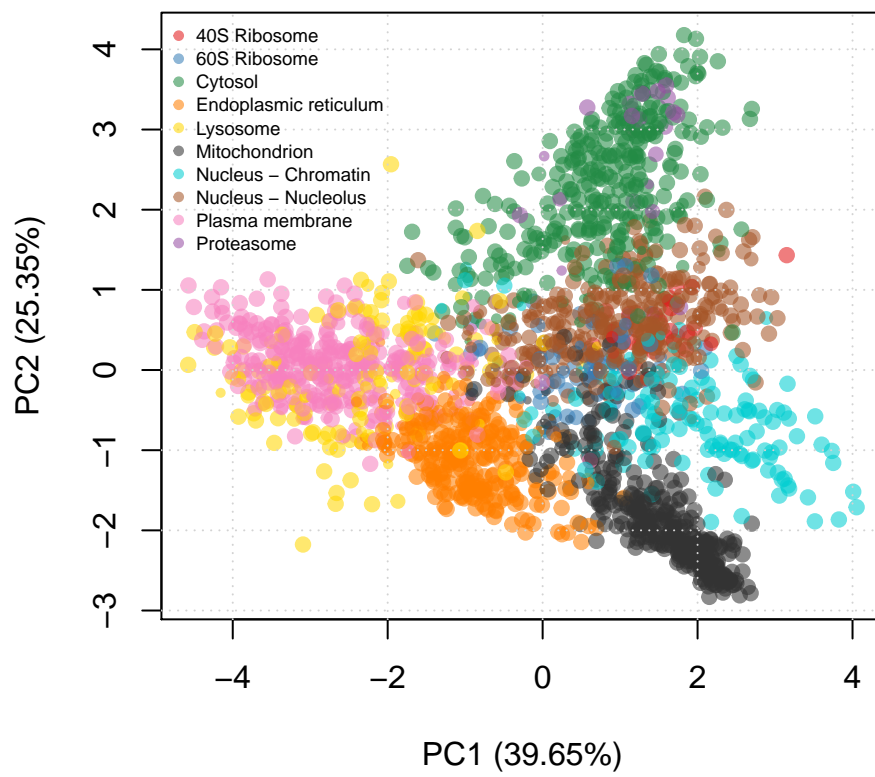


Figure 4: TAGM MAP allocations

4 Methods: *TAGM MCMC* a brief overview

The TAGM MCMC method allows a fully Bayesian analysis of spatial proteomics datasets. It employs a collapsed Gibbs sampler to obtain samples from the posterior distribution of localisation probabilities, providing a rich analysis of the data. This section demonstrates the advantage of taking a Bayesian approach and the biological information that can be extracted from this analysis.

Since our audience is unlikely to be versed in Bayesian methodology, we explain some of the key ideas for a more complete understanding. Firstly, MCMC based inference contrasts with MAP based inference in that it produces *samples* from the posterior distribution of localisation probabilities. Hence, we do not just have a single estimate for each quantity but a distribution of estimates. MCMC methods are a large class of algorithms used to sample from a probability distribution, in our case the posterior distribution of the parameters (Gilks, Richardson, and Spiegelhalter 1995). They design a Markov-chain; that is, a random sequence of events where the probability of the next event only depends on the current state, which, after convergence, obtains samples from the posterior distribution. A specific example of an MCMC algorithm is the Gibbs sampler, which can be applied when the parameters are conditionally conjugate. Often one can perform Rao-Blackwellisation, a method to reduce posterior variance, to obtain a collapsed Gibbs sampler (Casella and Robert 1996). Once one has obtained samples from the posterior distribution, we can estimate the true mean of the posterior distribution by simply taking the mean of the samples. In a similar fashion, we can obtain other summaries of the posterior distribution.

A schematic of MCMC sampling is provided in figure 5 to aid understanding. Proteins, coloured blue, are visualised along two variables of the data. Probability ellipses representing contours of a probability distribution matching the distribution of the proteins are overlaid. We now wish to obtain samples from this distribution. The MCMC algorithm is initialised with a starting location, then at each iteration a new value is proposed. These proposed values are either accepted or rejected (according to a carefully computed acceptance probability) and over many iterations the algorithm converges and this recipe produces samples from the desired distribution. A large portion of the earlier samples may not reflect the true distribution, because the MCMC sampler has yet to converge. These early samples are usually discarded and this is referred to informally as burnin. The next state of the algorithm depends on its current state and this leads to auto-correlation in the samples. To suppress this auto-correlation, we only retain every r^{th} sample. This is known as thinning. The details of burnin and thinning are further detailed in later sections.

The TAGM MCMC method is computationally intensive and requires at least modest processing power. Leaving the MCMC algorithm to run overnight on a modern desktop is usually sufficient, however this, of course, depends on the exact system properties. Do not expect the analysis to finish in a couple of hours on a medium specification laptop, for example.

To demonstrate the class structure and expected outputs of the TAGM MCMC method, we run a brief analysis on the a subsect (400 randomly chosen proteins) of the `tan2009r1` dataset from the `pRolocdata` purely for illustration. This is to provide a bare bones analysis of these data without being held back by computational requirements. We perform a complete demonstration and provide precise details of the analysis of the stem cell dataset considered above in the next section.

```
set.seed(1)
data(tan2009r1)
tan2009r1 <- tan2009r1[sample(nrow(tan2009r1), 400), ]
```

The first step is to run a few MCMC chains (below we use only 2) for a few iterations (three below, but typically in the order of ten thousands) of the algorithm using the `tagmMcmcTrain` function. This function will generate a object of class `MCMCParams`.

```
p <- tagmMcmcTrain(object = tan2009r1, numIter = 3,
                  burnin = 1, thin = 1, numChains = 2)
p

## Object of class "MCMCParams"
```

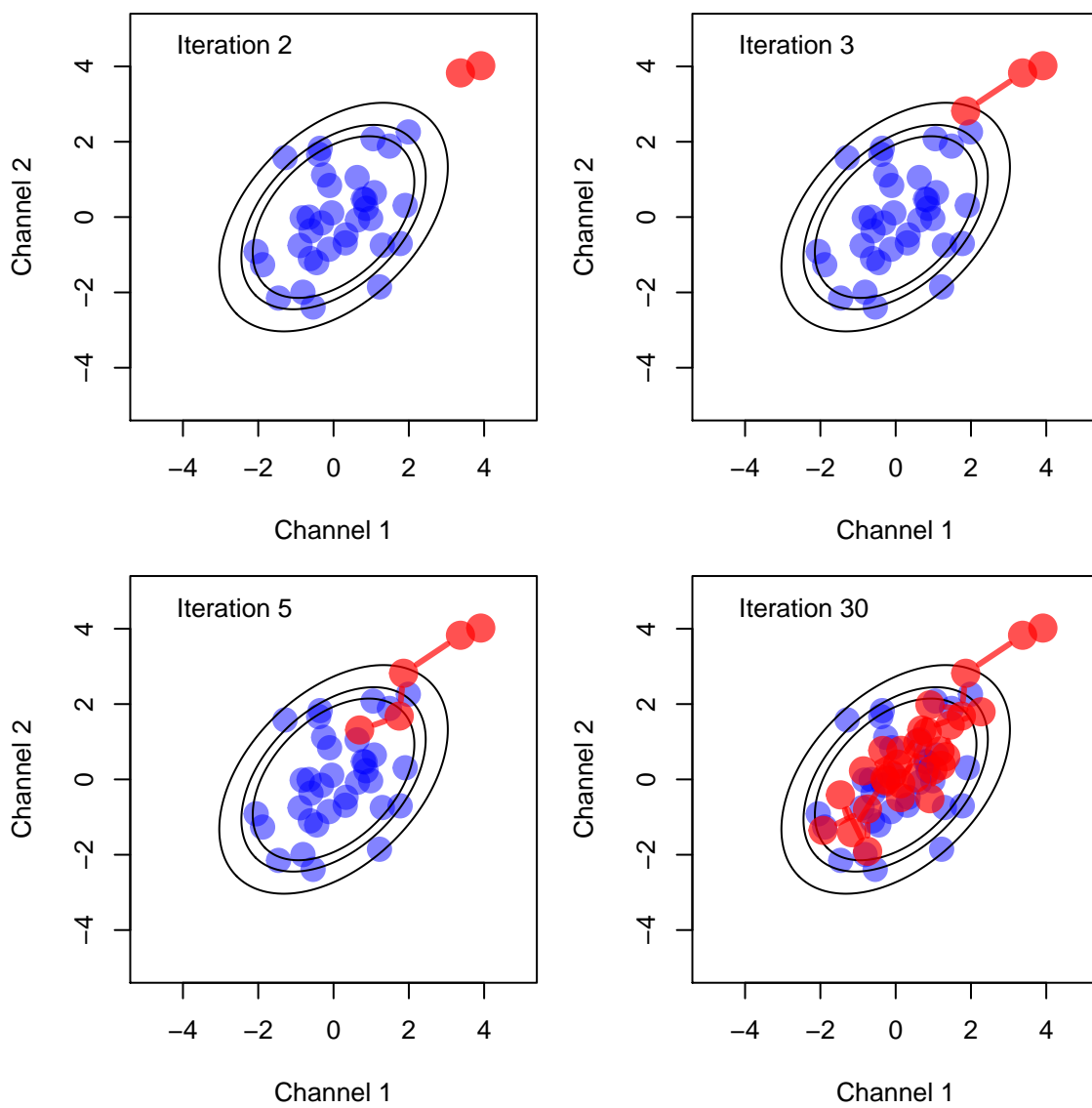


Figure 5: A schematic figure of MCMC sampling

```
## Method: TAGM.MCMC
## Number of chains: 2
```

Information for each MCMC chain is contained within the chains slot. If needed, this information can be accessed manually. The function `MCMCProcess` processed the `MCMCParams` object and populates the summary slot.

```
p <- tagmMcmcProcess(p)
p
```

```
## Object of class "MCMCParams"
## Method: TAGM.MCMC
## Number of chains: 2
## Summary available
```

The summary slot has now been populated to include basic summaries of the MCMC chains, such as organelle allocations and localisation probabilities. Protein information can be appended to the feature columns of the `MSnSet` by using the `tagmPredict` function, which extracts the required information from the summary slot of the `MCMCParams` object.

```
res <- tagmPredict(object = tan2009r1, params = p)
```

One can now access new features variables:

- `tagm.mcmc.allocation`: the TAGM MCMC prediction for the most likely protein sub-cellular annotation.

```
table(fData(res)$tagm.mcmc.allocation)
```

```
##
## Cytoskeleton      ER      Golgi      Lysosome mitochondrion
##           11      98      18      10      40
## Nucleus  Peroxisome      PM  Proteasome  Ribosome 40S
##           25      3      105      29      31
## Ribosome 60S
##           30
```

- `tagm.mcmc.probability`: the mean posterior probability for the protein sub-cellular allocations.

```
summary(fData(res)$tagm.mcmc.probability)
```

```
##   Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
## 0.3569 0.8788 0.9881 0.9049 1.0000 1.0000
```

As well as other useful summaries of the MCMC methods:

- `tagm.mcmc.outlier` the posterior probability for the protein to belong to the outlier component.
- `tagm.mcmc.probability.lowerquantile` and `tagm.mcmc.probability.upperquantile` are the lower and upper boundaries to the equi-tailed 95% credible interval of `tagm.mcmc.probability`.
- `tagm.mcmc.mean.shannon` a Monte-Carlo averaged shannon entropy, which is a measure of uncertainty in the allocations.

5 Methods: *TAGM MCMC* the details

This section explains how to manually manipulate the MCMC output of the TAGM model. In the code chunk below, we load the pre-computed TAGM MCMC model. The data file `e14tagm.rda` is available

online¹ and is not directly loaded into this package for size. The file itself is around 500mb, which is too large to directly load into a package.

```
load("e14Tagm.rda")
```

The following code, which is not evaluated dynamically, was used to produce the `tagmE14 MCMCParams` object. We run the MCMC algorithm for 20000 iterations with 10000 iterations discarded for burnin. We then thin the chain by 20. We ran 6 chains in parallel and so we obtain 500 samples for each of the 6 chains, totalling 3000 samples. The resulting file is assumed to be in our working directory.

```
e14Tagm <- tagmMcmcTrain(E14TG2aR,  
                        numIter = 20000,  
                        burnin = 10000,  
                        thin = 20,  
                        numChains = 6)
```

Manually inspecting the object, we see that it is a `MCMCParams` object with 6 chains.

```
e14Tagm  
  
## Object of class "MCMCParams"  
## Method: TAGM.MCMC  
## Number of chains: 6
```

5.1 Data exploration and convergence diagnostics

Assessing whether or not an MCMC algorithm has converged is challenging. Assessing and diagnosing convergence is an active area of research and throughout the 1990s many approaches were proposed (Geweke 1992; Andrew Gelman and Rubin 1992; Roberts and Smith 1994; Brooks and Gelman 1998). A converged MCMC algorithm should be oscillating rapidly around a single value with no monotonicity. We provide a more detailed exploration of this issue, but the readers should bear in mind that the methods provided below are diagnostics and cannot guarantee success. We direct readers to several important works in the literature discussing the assessment of convergence. Users that do not assess convergence and base their downstream analysis on unconverged chains are likely to obtain poor quality results.

We first assess convergence using a parallel chains approach. We find producing multiple chains is beneficial not only for computational advantages but also for analysis of convergence of our chains.

```
## Get number of chains  
nChains <- length(e14Tagm)  
nChains
```

```
## [1] 6
```

The following code chunks sets up a manual convergence diagnostic check. We make use of objects and methods in the package *coda* to perform this analysis (Plummer et al. 2006). Our function below automatically coerces our objects into *coda* for ease of analysis. We first calculate the total number of outliers at each iteration of each chain and, if the algorithm has converged, this number should be the same (or very similar) across all 6 chains.

```
## Convergence diagnostic to see if more we need to discard any  
## iterations or entire chains: compute the number of outliers for  
## each iteration for each chain  
out <- mcmc_get_outliers(e14Tagm)
```

We can observe this from the trace plots and histograms for each MCMC chain (figure 6). Unconverged chains should be discarded from downstream analysis.

¹<https://drive.google.com/open?id=1zozntDhE6YZ-q8wjtQ-lxZ66EEszOGYi>

```
## Using coda S3 objects to produce trace plots and histograms
par(mfrow = c(6, 2))
for (i in seq_len(nChains))
  plot(out[[i]], main = paste("Chain", i), auto.layout = FALSE, col = i)
```

Chains 3, 5 and 6 oscillate around an average of 153, with rapid back and forth oscillations. Chain 2 should be immediately discarded, since it has a large jump in the chain with clearly skewed histogram. The other two chains oscillate differently with contrasting quantiles to the 3 chains in agreement, suggesting these chains have yet to converge. We can use the *coda* package to produce summaries of our chains. Here is the *coda* summary for the third chain.

```
## Chains average around 153 outliers
summary(out[[3]])

##
## Iterations = 1:500
## Thinning interval = 1
## Number of chains = 1
## Sample size per chain = 500
##
## 1. Empirical mean and standard deviation for each variable,
##    plus standard error of the mean:
##
##           Mean           SD      Naive SE Time-series SE
##       153.4520       14.0771       0.6295       0.6820
##
## 2. Quantiles for each variable:
##
##  2.5%   25%   50%   75%  97.5%
##   127   144   153   162   183
```

5.1.1 Applying the Gelman diagnostic

Thus far, our analysis appears promising. Three separate chains oscillate around an average of 153 outliers and there is no observed monotonicity in our output. However, for a more rigorous and unbiased analysis of convergence we can calculate the Gelman diagnostic using the *coda* package (Andrew Gelman and Rubin 1992; Brooks and Gelman 1998). This statistics is often referred to as \hat{R} or the potential scale reduction factor. The idea of the Gelman diagnostics is to compare the inter and intra chain variances. The ratio of these quantities should be close to one. The actual statistics computed is more complicated, but we do not go deeper here and a more detailed and in depth discussion can be found in the references. The *coda* package also reports the 95% upper confidence interval of the \hat{R} statistic. In this case, our samples are normally distributed (see traces on the right in figure 6). The *coda* package allows for transformations to improve normality of the data, and in some cases we set the `transform` argument to apply log transformation. Andrew Gelman and Rubin (1992) suggest that chains with \hat{R} value of less than 1.2 are likely to have converged.

```
gelman.diag(out, transform = FALSE)

## Potential scale reduction factors:
##
##      Point est. Upper C.I.
## [1,]       1.14       1.32

gelman.diag(out[c(1,3,4,5,6)], transform = FALSE)

## Potential scale reduction factors:
```

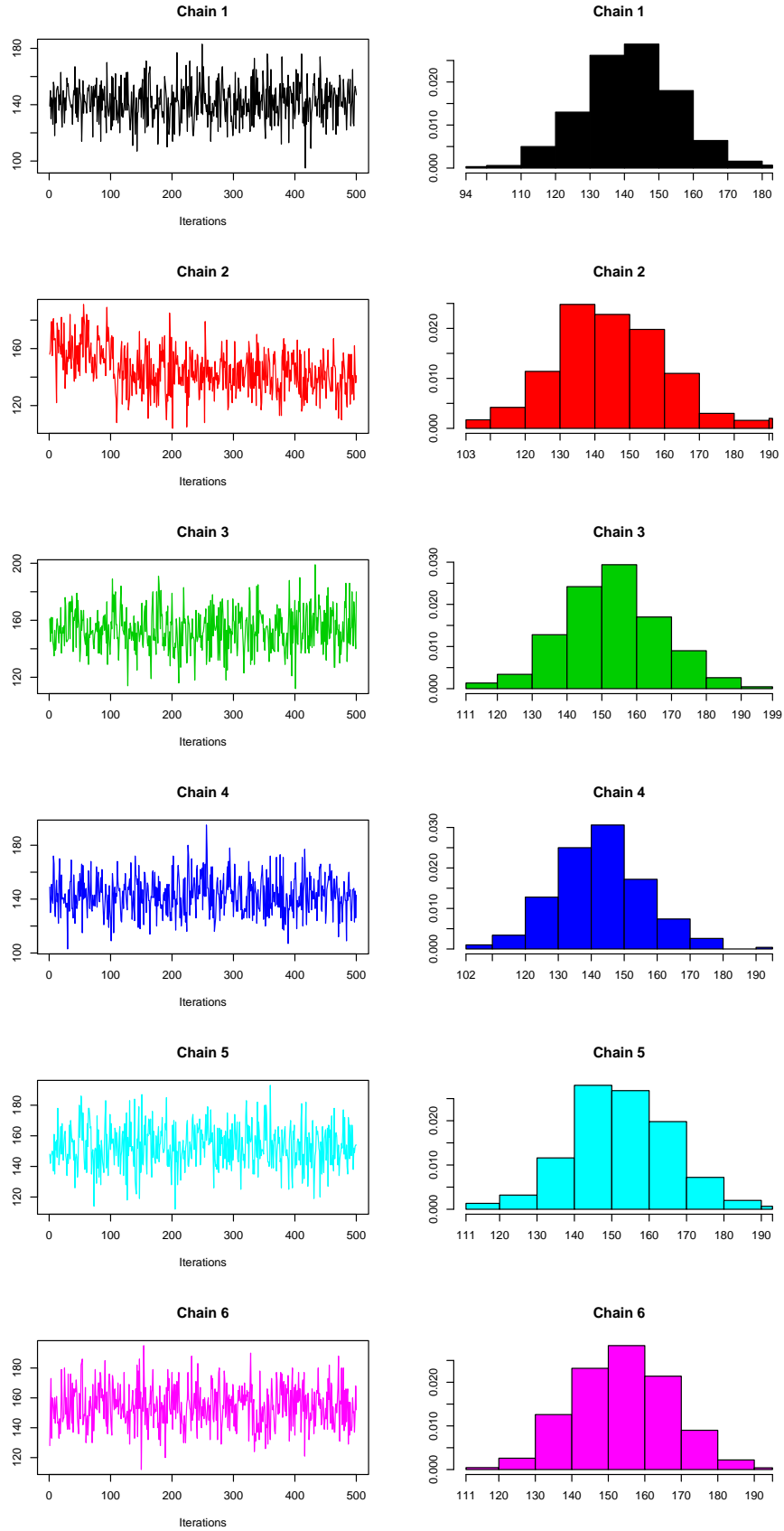


Figure 6: Trace (left) and density (right) of the 6 MCMC chains.

```
##
##      Point est. Upper C.I.
## [1,]      1.13      1.31
```

```
gelman.diag(out[c(3,5,6)], transform = FALSE)
```

```
## Potential scale reduction factors:
```

```
##
##      Point est. Upper C.I.
## [1,]      1      1.01
```

In all cases, we see that the Gelman diagnostic for convergence is < 1.2 . However, the upper confidence interval is 1.32 when all chains are used; 1.31 when chain 2 is removed and when chains 1, 2 and 4 are removed the upper confidence interval is 1.01 indicating that the MCMC algorithm for chains 3,5 and 6 might have converged.

We can also look at the Gelman diagnostics statistics for groups or pairs of chains. The first line below compute the Gelman diagnostic across the first three chains, whereas the second calculates between chain 3 and chain 5.

```
gelman.diag(out[1:3], transform = FALSE) # the upper C.I is 1.62
```

```
## Potential scale reduction factors:
```

```
##
##      Point est. Upper C.I.
## [1,]      1.22      1.62
```

```
gelman.diag(out[c(3,5)], transform = TRUE) # the upper C.I is 1.01
```

```
## Potential scale reduction factors:
```

```
##
##      Point est. Upper C.I.
## [1,]      1.01      1.01
```

To assess another summary statistic, we can look at the mean component allocation at each iteration of the MCMC algorithm and as before we produce trace plots of this quantity (figure 7).

```
meanAlloc <- mcmc_get_meanComponent(e14Tagm)
```

```
par(mfrow = c(6, 2))
for (i in seq_len(nChains))
  plot(meanAlloc[[i]], main = paste("Chain", i), auto.layout = FALSE, col = i)
```

As before we can produce summaries of the data.

```
summary(meanAlloc[[1]])
```

```
##
## Iterations = 1:500
## Thinning interval = 1
## Number of chains = 1
## Sample size per chain = 500
##
## 1. Empirical mean and standard deviation for each variable,
##    plus standard error of the mean:
##
##      Mean      SD      Naive SE Time-series SE
## 5.686713 0.059112 0.002644      0.002644
##
```

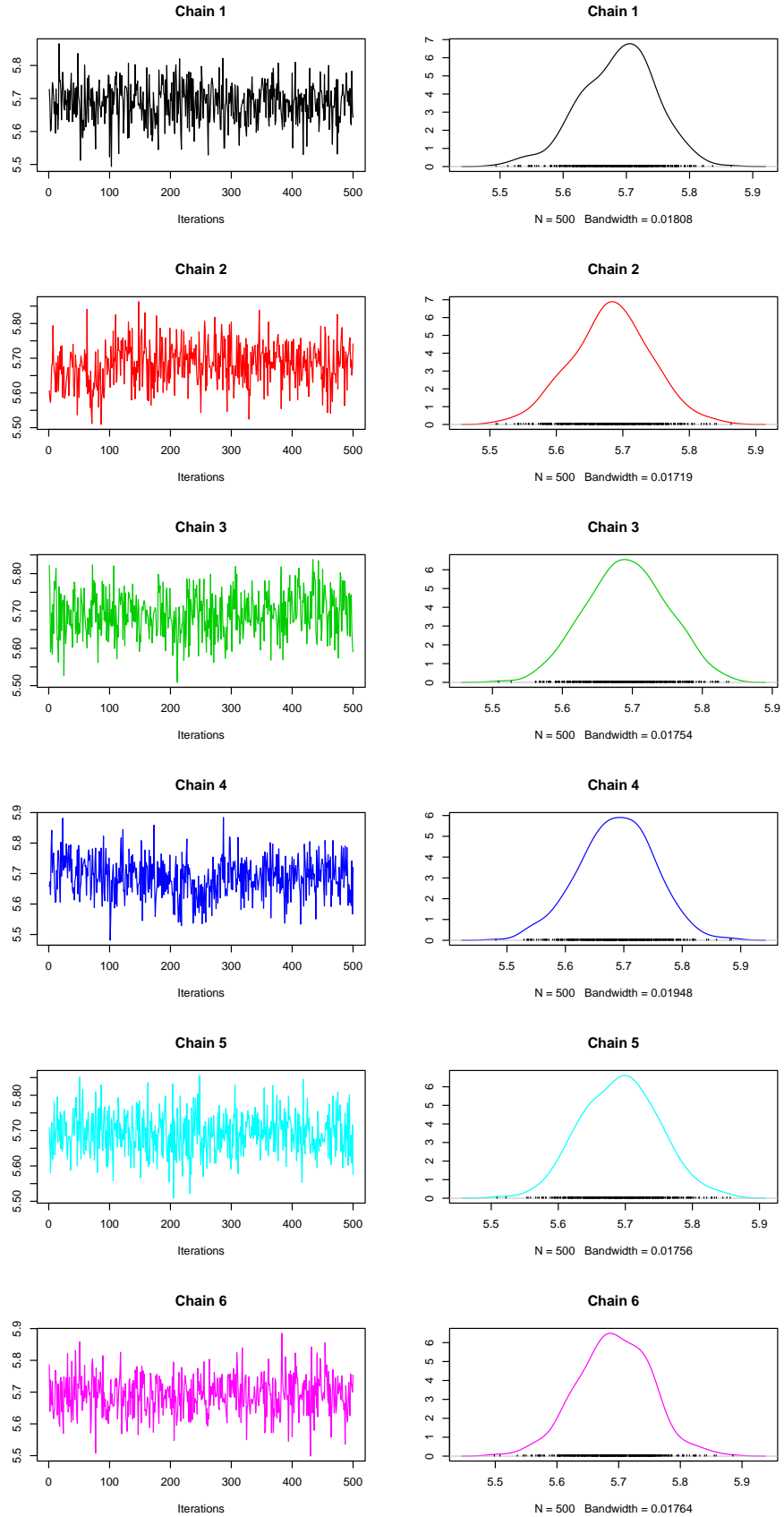


Figure 7: Trace (left) and density (right) of the mean component allocation 6 MCMC chains.


```
## 2. Quantiles for each variable:
##
## 2.5% 25% 50% 75% 97.5%
## 5.552 5.646 5.692 5.728 5.795
```

We can already observe that there are some slight difference between these chains this raises suspicion that some of the chains may not have converged. For example each chains appears to be oscillating around 5.7, but chains 2 and 4 have clear bumps in the their trace plots. For a more quantitative analysis, we again apply the Gelman diagnostics to these summaries.

```
gelman.diag(meanAlloc)
```

```
## Potential scale reduction factors:
##
##      Point est. Upper C.I.
## [1,]          1          1.01
```

The above values are close to 1 and so there are no significant difference between the chains. As observed previously, chains 2 and 4 look quite different from the other chains and so we recalculate the diagnostic excluding these chains. The computed Gelman diagnostic below suggest that chains 3, 5 and 6 have converged and that we should discard chains 1, 2 and 4 from further analysis.

```
gelman.diag(meanAlloc[c(3,5,6)])
```

```
## Potential scale reduction factors:
##
##      Point est. Upper C.I.
## [1,]          1          1
```

For a further check, we can look at the mean outlier probability at each iteration of the MCMC algorithm and again computing the Gelman diagnostics between chains 4, 5 and 6. An \hat{R} statistics of 1 is indicative of convergence, since it is less than the recommended value of 1.2.

```
meanoutProb <- mcmc_get_meanoutliersProb(e14Tagm)
gelman.diag(meanoutProb[c(3, 5, 6)])
```

```
## Potential scale reduction factors:
##
##      Point est. Upper C.I.
## [1,]          1          1.01
```

5.1.2 Applying the Geweke diagnostic

Along with the Gelman diagnostics, which uses parallel chains, we can also apply a single chain analysis using the Geweke diagnostic. The Geweke diagnostic tests to see whether the mean calculated from the first 10% of iterations is significantly different from the mean calculated from the last 50% of iterations. If they are significantly different, at say a level 0.01, then this is evidence that particular chains has not converged. The following code chunk calculates the Geweke diagnostic for each chain on the summarising quantities we have previously computed.

```
geweke_test(out)
```

```
##          chain 1      chain 2 chain 3      chain 4      chain 5      chain 6
## z.value 0.5749775 8.816632e+00 0.470203 -0.3204500 -0.6270787 -0.7328168
## p.value 0.5653065 1.179541e-18 0.638210 0.7486272 0.5306076 0.4636702
```

```
geweke_test(meanAlloc)
```

```
##          chain 1      chain 2      chain 3      chain 4      chain 5      chain 6
## z.value 1.1952967 -3.3737051063 -1.2232102 2.48951993 0.3605882 -0.1358850
## p.value 0.2319711 0.0007416377 0.2212503 0.01279157 0.7184073 0.8919122
```

```
geweke_test(meanoutProb)
```

```
##          chain 1      chain 2      chain 3      chain 4      chain 5      chain 6
## z.value 0.1785882 1.205500e+01 0.6189637 -0.5164987 -0.2141086 -0.02379004
## p.value 0.8582611 1.825379e-33 0.5359403 0.6055062 0.8304624 0.98102008
```

The first test suggest chain 2 has not converged, since the p-value is less than 10^{-10} suggesting that the mean in the first 10% of iterations is significantly different from those in the final 50%. Moreover, the second test and third tests also suggest chain 2 has not converged. Furthermore, for the second test chain 4 has a marginally small p-value and thus further evidence that this chain is of low quality. These convergence diagnostics are not limited to the quantities we have computed here and further diagnostics can be perform on any summary of the data.

An important question to consider is whether removing an early portion of the chain might lead to improvement of the convergence diagnostics. This might be particularly relevant if a chain converges some iterations after our originally specified `burnin`. For example let us take the second Geweke test above, which suggested chains 2 and 4 had not converged and see if discarding the initial 10% of the chain improves the statistic. The function below removes 50 samples, informally known as `burnin`, from the beginning of each chain and the output shows that we now have 450 samples in each chain.

```
burne14Tagm <- mcmc_burn_chains(e14Tagm, 50)
chains(burne14Tagm)
```

```
## Object of class "MCMCChains"
## Number of chains: 6
```

```
chains(burne14Tagm)[[4]]
```

```
## Object of class "MCMCChain"
## Number of components: 10
## Number of proteins: 1663
## Number of iterations: 450
```

The following function recomputes the number of outliers in each chain at each iteration of each Markov-chain.

```
newout <- mcmc_get_outliers(burne14Tagm)
```

The code chunk below compute the Geweke diagnostic for this new truncated chain and demonstrates that chain 4 has an improved Geweke diagnostic, whilst chain 2 does not. Thus, in practice, it maybe useful to remove iterations from the beginning of the chain. However, as chain 4 did not pass the Gelman diagnostics we still discard it from downstream analysis.

```
geweke_test(newout)
```

```
##          chain 1      chain 2      chain 3      chain 4      chain 5      chain 6
## z.value -0.1455345 6.379618e+00 -1.6392215 0.3836940 0.1241201 0.6654703
## p.value 0.8842889 1.775298e-10 0.1011671 0.7012053 0.9012202 0.5057497
```

5.2 Processing converged chains

Having made an assessment of convergence, we decide to discard chains 1, 2 and 4 from any further analysis. The code chunk below remove these chains and creates a new object to store the converged chains.

```
removeChain <- c(1, 2, 4) # The chains to be removed
e14Tagm_converged <- e14Tagm[-removeChain] # Create new object
```

The `MCMCParams` object can be large and therefore if we have a large number of samples we may want to subsample our chain, informally known as thinning, to reduce the number of samples. Thinning also has another purpose. We may desire independent samples from our posterior distribution but the MCMC algorithm produces autocorrelated samples. Thinning can be applied to reduce the autocorrelation between samples. The code chunk below, which is not evaluated demonstrates retaining every 5th iteration. Recall that we thinned by 20 when we first ran the MCMC algorithm.

```
e14Tagm_converged_thinned <- mcmc_thin_chains(e14Tagm_converged, freq = 5)
```

We initially ran 6 chains and, after having made an assessment of convergence, we decided to discard 3 of the chains. We desire to make inference using samples from all 3 chains, since this leads to better posterior estimates. In their current class structure all the chains are stored separately, so the following function pools all sample for all chains together to make a single longer chain with all samplers. Pooling a mixture of converged and unconverged chains is likely to lead to poor quality results.

```
e14Tagm_converged_pooled <- mcmc_pool_chains(e14Tagm_converged)
e14Tagm_converged_pooled
```

```
## Object of class "MCMCParams"
## Method: TAGM.MCMC
## Number of chains: 1
```

```
e14Tagm_converged_pooled[[1]]
```

```
## Object of class "MCMCChain"
## Number of components: 10
## Number of proteins: 1663
## Number of iterations: 1500
```

To populate the summary slot of the converged and pooled chain, we can use the `tagmMcmcProcess` function. As we can see from the object below a summary is now available. The information now available in the summary slot was detailed in the previous section. We note that if there is more than 1 chain in the `MCMCParams` object then the chains are automatically pooled to compute the summaries.

```
e14Tagm_converged_pooled <- tagmMcmcProcess(e14Tagm_converged_pooled)
e14Tagm_converged_pooled
```

```
## Object of class "MCMCParams"
## Method: TAGM.MCMC
## Number of chains: 1
## Summary available
```

To create new feature columns in the `MSnSet` and appened the summary information, we apply the `tagmPredict` function. The `probJoint` argument indicates whether or not to add probabilistic information for all organelles for all proteins, rather than just the information for the most probable organelle. The outlier probabilities are also return by default, but users can change this using the `probOutlier` argument.

```
E14TG2aR <- tagmPredict(object = E14TG2aR,
                        params = e14Tagm_converged_pooled,
                        probJoint = TRUE)
head(fData(E14TG2aR))
```

```
##          Uniprot.ID UniprotName
## Q62261      Q62261 SPTB2_MOUSE
## Q9JHU4      Q9JHU4 DYHC1_MOUSE
## Q9QXS1      Q9QXS1 PLEC_MOUSE
## P16546      P16546 SPTA2_MOUSE
```

```

## Q69ZN7      Q69ZN7  MYOF_MOUSE
## P30999      P30999  CTND1_MOUSE
##
##                               Protein.Description Peptides PSMs
## Q62261 Spectrin beta chain, brain 1 (multiple isoforms)      42  42
## Q9JHU4      Cytoplasmic dynein 1 heavy chain 1              33  33
## Q9QXS1      Isoform PLEC-1I of Plectin                      33  33
## P16546 Spectrin alpha chain, brain (multiple isoforms)      32  32
## Q69ZN7      Myoferlin (multiple isoforms)                  28  28
## P30999      Catenin delta-1 (multiple isoforms)             24  24
##
##      GOannotation markers.orig      markers      tagm.map.allocation
## Q62261      PLM-SKE      unknown      unknown Endoplasmic reticulum
## Q9JHU4      SKE      unknown      unknown Nucleus - Chromatin
## Q9QXS1      unknown      unknown      unknown Plasma membrane
## P16546      PLM-SKE-CYT      unknown      unknown Nucleus - Chromatin
## Q69ZN7      VES      unknown      unknown Plasma membrane
## P30999      PLM-NUC      PLM Plasma membrane      Plasma membrane
##
##      tagm.map.probability tagm.map.outlier tagm.mcmc.allocation
## Q62261      0.5710958      0 Endoplasmic reticulum
## Q9JHU4      1.0000000      0 Nucleus - Chromatin
## Q9QXS1      0.9999997      0 Proteasome
## P16546      0.9314100      0 Endoplasmic reticulum
## Q69ZN7      0.9995585      0 Plasma membrane
## P30999      1.0000000      0 Plasma membrane
##
##      tagm.mcmc.probability tagm.mcmc.probability.lowerquantile
## Q62261      0.5765793      0.0020296117
## Q9JHU4      0.9738206      0.7594516090
## Q9QXS1      0.4957129      0.0002886457
## P16546      0.5214374      0.0014041362
## Q69ZN7      0.9997025      0.9981794326
## P30999      1.0000000      1.0000000000
##
##      tagm.mcmc.probability.upperquantile tagm.mcmc.mean.shannon
## Q62261      0.9992504      0.201623229
## Q9JHU4      0.9998822      0.081450206
## Q9QXS1      0.9947100      0.447665536
## P16546      0.9946959      0.252833750
## Q69ZN7      0.9999954      0.002395147
## P30999      1.0000000      0.000000000
##
##      tagm.mcmc.outlier tagm.mcmc.joint.40S Ribosome
## Q62261      2.547793e-01      4.401228e-10
## Q9JHU4      3.335134e-05      1.936225e-18
## Q9QXS1      6.423799e-01      2.213861e-07
## P16546      2.119112e-01      1.576023e-09
## Q69ZN7      7.274103e-06      3.510523e-22
## P30999      0.000000e+00      0.000000e+00
##
##      tagm.mcmc.joint.60S Ribosome tagm.mcmc.joint.Cytosol
## Q62261      2.778620e-07      2.650861e-12
## Q9JHU4      1.645727e-21      1.887645e-17
## Q9QXS1      1.495170e-01      9.062280e-09
## P16546      3.150122e-06      1.471329e-08
## Q69ZN7      5.152312e-16      2.063009e-24
## P30999      0.000000e+00      0.000000e+00
##
##      tagm.mcmc.joint.Endoplasmic reticulum tagm.mcmc.joint.Lysosome
## Q62261      5.765793e-01      1.108757e-11
## Q9JHU4      1.548053e-17      5.577415e-24

```

## Q9QXS1	1.768681e-04	1.150706e-04
## P16546	5.214374e-01	3.687975e-09
## Q69ZN7	8.397027e-09	2.974966e-04
## P30999	0.000000e+00	0.000000e+00
## tagm.mcmc.joint.Mitochondrion	tagm.mcmc.joint.Nucleus	- Chromatin
## Q62261	5.020528e-08	4.231731e-01
## Q9JHU4	2.835919e-22	9.738206e-01
## Q9QXS1	5.832273e-19	7.920397e-03
## P16546	4.522032e-08	4.776913e-01
## Q69ZN7	6.143974e-39	4.872032e-21
## P30999	0.000000e+00	0.000000e+00
## tagm.mcmc.joint.Nucleus	- Nucleolus	tagm.mcmc.joint.Plasma membrane
## Q62261	1.279255e-05	1.914808e-11
## Q9JHU4	2.617943e-02	3.514851e-29
## Q9QXS1	1.130580e-05	3.465462e-01
## P16546	3.448558e-05	2.489652e-07
## Q69ZN7	7.042891e-30	9.997025e-01
## P30999	0.000000e+00	1.000000e+00
## tagm.mcmc.joint.Proteasome		
## Q62261	2.345204e-04	
## Q9JHU4	7.841425e-11	
## Q9QXS1	4.957129e-01	
## P16546	8.333595e-04	
## Q69ZN7	1.003778e-10	
## P30999	0.000000e+00	

Aside: *Priors*

Bayesian analysis requires users to specify prior information about the parameters. This potentially appears a challenging task; however, good default options are often possible. Should expert information be available for any of these priors then the users should provide this, else we have found that the default choices work well in practice. The priors also provide regularisation and shrinkage to avoid overfitting. Given enough data the likelihood overwhelms the prior and little inference is based on the prior. There is little guidance in the literature on how to choose priors or even what they mean for the data analysis.

We place a normal inverse-Wishart prior on the normally distributed mixture components. The normal inverse-Wishart prior has 4 hyperparameters to be chosen to specify the prior beliefs in the mean and covariance of these mixtures. These are the prior mean μ_0 expressing the prior location of each organelle; a prior shrinkage λ_0 a scalar expressing uncertainty in the prior mean; the prior degrees of freedom ν_0 and a scale prior S_0 on the covariance. Together ν_0 and S_0 specify the prior variability on organelle covariances. All components share the same prior information.

The default options for these hold little information and are based on choice recommended by (Fraley and Raftery 2005). The prior mean μ_0 is set to mean of the data. λ_0 is set to be 0.01 meaning some uncertainty in the covariance is propagated to the mean, increasing λ_0 increase shrinkage towards the prior. ν_0 is set to the number of feature variables plus 2, which is the smallest integer value that ensures a finite covariance matrix. The prior scale matrix S_0 is set to

$$S_0 = \frac{\text{diag}(\frac{1}{n} \sum (X - \bar{X})^2)}{K^{1/D}}, \quad (1)$$

and represents a diffuse prior on the covariance. Another good choice which is often used is a constant multiple of the identity matrix. The prior for the Dirichlet distribution concentration parameters β_0 is set to 1 for each organelle. This represents a symmetric belief about the number of proteins allocated to

each organelle. Another reasonable choice would be the non-informative Jeffery's prior for the Dirichlet hyperparameter, which sets `beta0` is 0.5 for each organelle. The prior weight for the outlier detection class is a $\mathcal{B}(u, v)$ distribution. The default for $u = 2$ and the default for $v = 10$. This represent the reasonable belief that $\frac{u}{u+v} = \frac{1}{6}$ proteins *a priori* might be an outlier and we believe is unlikely that more than 50% of proteins are outliers. Decreasing the value of v , represent more uncertainty about the number of protein that are outliers.

5.3 Analysis, visualisation and interpretation of results

Now that we have single pooled chain of samples from a converged MCMC algorithm, we can begin to analyse the results. Preliminary analysis includes visualising the allocated organelle and localisation probability of each protein to its most probable organelle, as shown on figure 8.

```
par(mfrow = c(1, 2))
plot2D(E14TG2aR, fcol = "tagm.mcmc.allocation",
       cex = fData(E14TG2aR)$tagm.mcmc.probability,
       main = "TAGM MCMC allocations")
addLegend(E14TG2aR, fcol = "markers",
          where = "topleft", ncol = 2, cex = 0.6)

plot2D(E14TG2aR, fcol = "tagm.mcmc.allocation",
       cex = fData(E14TG2aR)$tagm.mcmc.mean.shannon,
       main = "Visualising global uncertainty")
addLegend(E14TG2aR, fcol = "markers",
          where = "topleft", ncol = 2, cex = 0.6)
```

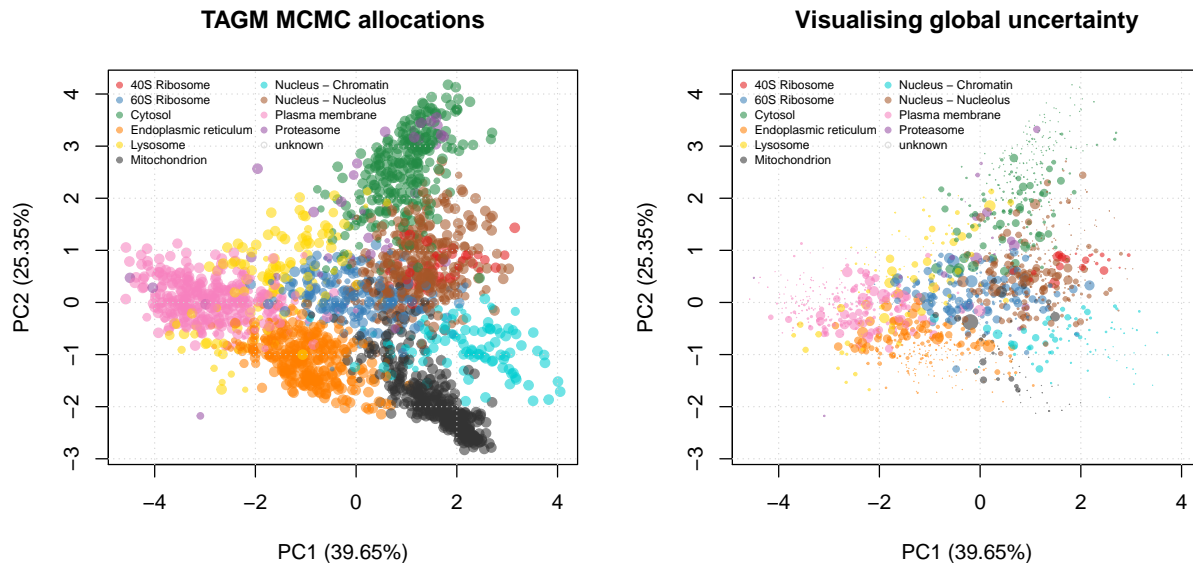


Figure 8: TAGM MCMC allocations. On the left, point size have been scaled based on allocation probabilities. On the right, the point size have been scaled based on the global uncertainty using the mean Shannon entropy.

We can visualise other summaries of the data including a Monte-Carlo averaged Shannon entropy, as show on figure 8 on the right. This is a measure of uncertainty and proteins with greater shannon entropy have more uncertainty in their localisation. We observe global patterns of uncertainty, particularly in areas where

organelle boundaries overlap. There are also regions of low uncertainty indicating little doubt about the localisation of these proteins.

We are also interested in the relationship between localisation probability to the most probable class and the Shannon entropy. Eventhough the two quantities are evidently correlated there is still considerable spread. Thus it is important to based inference not only on localisation probability but also a measure of uncertainty for example the Shannon entropy.

```
cls <- getStockcol()[as.factor(fData(E14TG2aR)$tagm.mcmc.allocation)]
plot(fData(E14TG2aR)$tagm.mcmc.probability,
     fData(E14TG2aR)$tagm.mcmc.mean.shannon,
     col = cls, pch = 19,
     xlab = "Localisation probability",
     ylab = "Shannon entropy")
addLegend(E14TG2aR, fcol = "markers",
          where = "topright", ncol = 2, cex = 0.6)
```

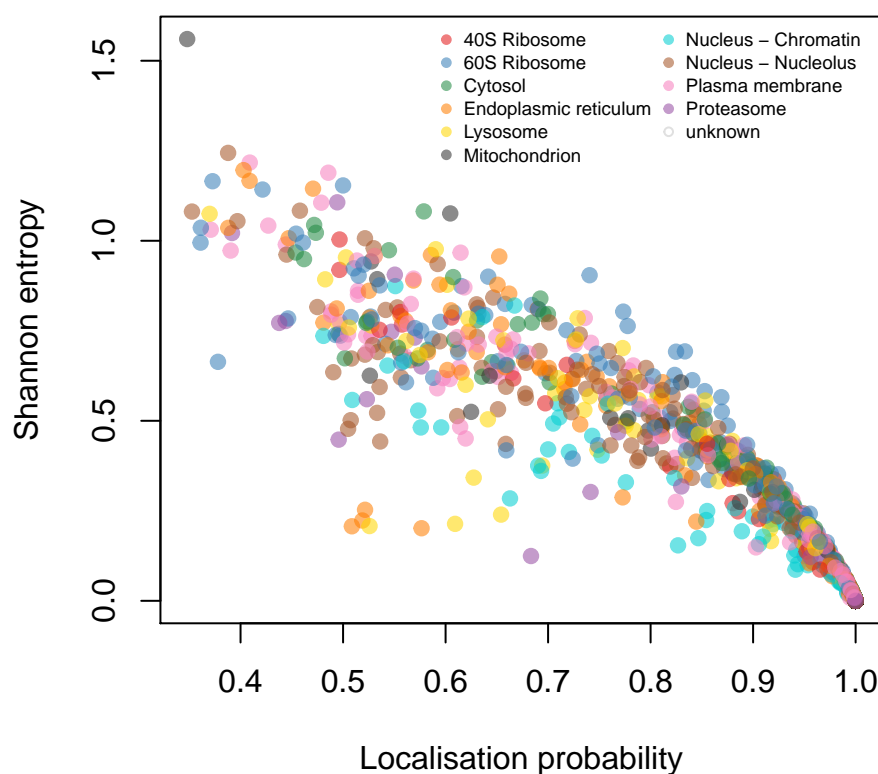


Figure 9: Shannon entropy and localisation probability.

Aside from global visualisation of the data, we can also interrogate each individual protein. As illustrated on figure 10, we can obtain the full posterior distribution of localisation probabilities for each protein from the `e14Tagm_converged_pooled` object. We can use the `plot` generic on the `MCMCParams` object to obtain a violin plot of the localisation distribution. Simply providing the name of the protein in the second argument produce the plot for that protein. The solute carrier transporter protein E9QMX3, also refered to as Slc15a1,

is most probably localised to plasma membrane in line with its role as a transmembrane transporter but also shows some uncertainty, potentially also localising to other compartments. The first violin plot visualises this uncertainty. The protein Q3V1Z5 is a supposed constituent of the 40S ribosome and has poor UniProt annotation with evidence only at the transcript level. From the plot below it is clear that Q3V1Z5 is a ribosomal associated protein, but its previous localisation has only been computationally inferred and here we provide experimental evidence of a ribosomal annotation. Thus, quantifying uncertainty recovers important additional annotations.

```
p1 <- plot(e14Tagm_converged_pooled, "E9QMX3")
p2 <- plot(e14Tagm_converged_pooled, "Q3V1Z5")
library(patchwork)
p1 / p2
```

6 Discussion

We have provided a workflow for the Bayesian analysis of spatial proteomics using the **pRoloc** and **MSnbase** software. We have demonstrated, in a step by step fashion, the challenges and advantages to taking a Bayesian approach to data analysis. We hope this workflow will help spatial proteomics practitioners to apply our method and inspires others to create detailed documentation for the Bayesian analysis of biological data.

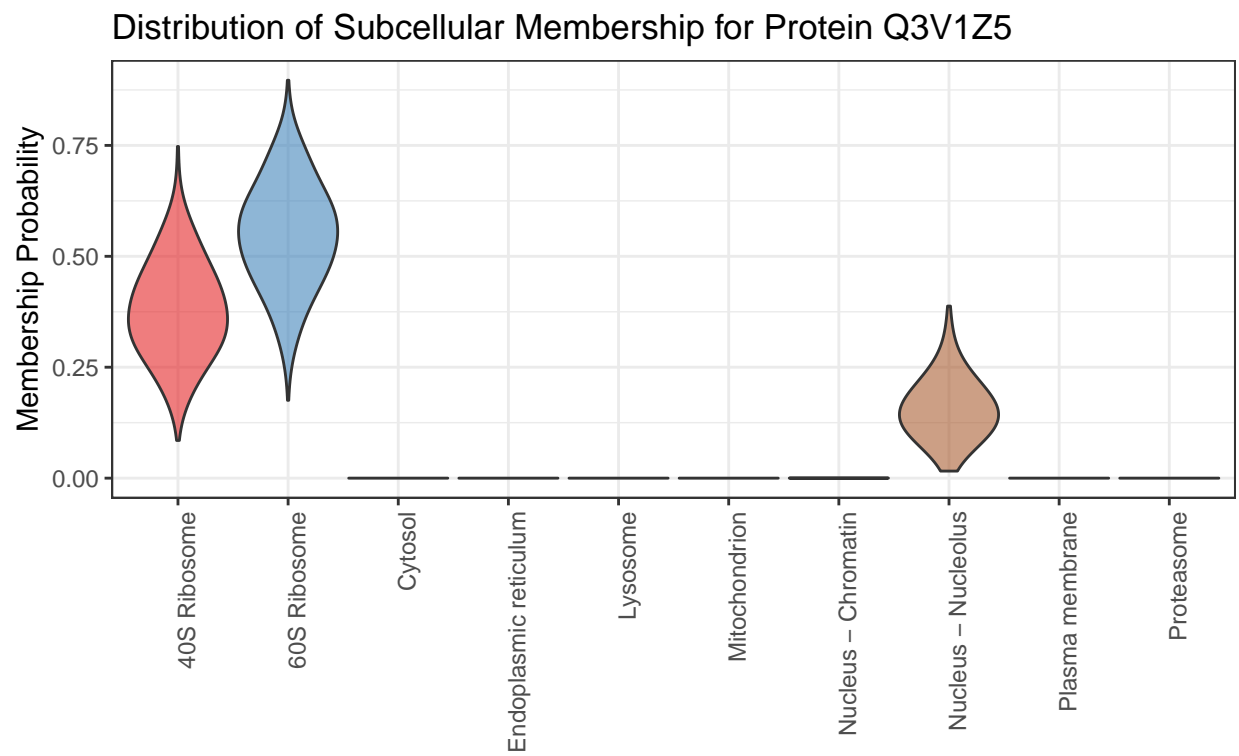
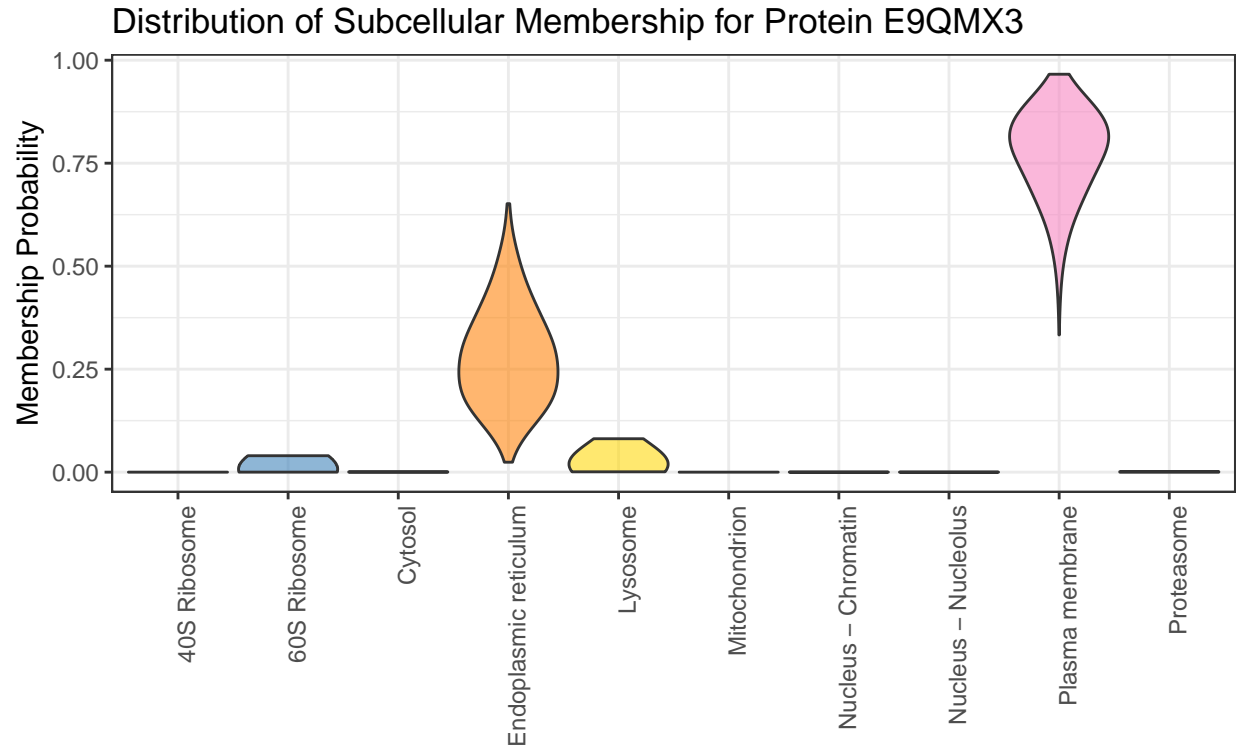


Figure 10: Full posterior distribution of localisation probabilities for individual proteins.

7 Session information

Below, we provide a summary of all packages and versions used to generate this document.

```
sessionInfo()
```

```
## R version 3.5.2 (2018-12-20)
## Platform: x86_64-w64-mingw32/x64 (64-bit)
## Running under: Windows 10 x64 (build 17134)
##
## Matrix products: default
##
## locale:
## [1] LC_COLLATE=English_United Kingdom.1252
## [2] LC_CTYPE=English_United Kingdom.1252
## [3] LC_MONETARY=English_United Kingdom.1252
## [4] LC_NUMERIC=C
## [5] LC_TIME=English_United Kingdom.1252
##
## attached base packages:
## [1] stats4      parallel  stats      graphics  grDevices  utils      datasets
## [8] methods     base
##
## other attached packages:
## [1] patchwork_0.0.1      pRolocdata_1.20.0    pRoloc_1.23.2
## [4] coda_0.19-2          mixtools_1.1.0       BiocParallel_1.16.5
## [7] MLInterfaces_1.62.0   cluster_2.0.7-1      annotate_1.60.0
## [10] XML_3.98-1.16         AnnotationDbi_1.44.0 IRanges_2.16.0
## [13] MSnbase_2.8.3         ProtGenerics_1.14.0  S4Vectors_0.20.1
## [16] mzR_2.16.1           Rcpp_1.0.0           Biobase_2.42.0
## [19] BiocGenerics_0.28.0
##
## loaded via a namespace (and not attached):
## [1] snow_0.4-3            plyr_1.8.4            igraph_1.2.2
## [4] lazyeval_0.2.1        splines_3.5.2          ggvis_0.4.4
## [7] crosstalk_1.0.0       ggplot2_3.1.0          digest_0.6.18
## [10] foreach_1.4.4         htmltools_0.3.6        viridis_0.5.1
## [13] gdata_2.18.0          magrittr_1.5           memoise_1.1.0
## [16] doParallel_1.0.14     sfsmisc_1.1-3          limma_3.38.3
## [19] recipes_0.1.4         gower_0.1.2           rda_1.0.2-2.1
## [22] lpSolve_5.6.13        prettyunits_1.0.2      colorspace_1.4-0
## [25] blob_1.1.1            xfun_0.4              dplyr_0.7.8
## [28] crayon_1.3.4          RCurl_1.95-4.11        hexbin_1.27.2
## [31] genefilter_1.64.0     bindr_0.1.1           impute_1.56.0
## [34] survival_2.43-3       iterators_1.0.10       glue_1.3.0
## [37] gtable_0.2.0          ipred_0.9-8            zlibbioc_1.28.0
## [40] kernlab_0.9-27        prabclus_2.2-7         DEoptimR_1.0-8
## [43] scales_1.0.0          vsn_3.50.0            mvtnorm_1.0-8
## [46] DBI_1.0.0             viridisLite_0.3.0     xtable_1.8-3
## [49] progress_1.2.0        bit_1.1-14            proxy_0.4-22
## [52] mclust_5.4.2          preprocessCore_1.44.0  lava_1.6.4
## [55] prodlim_2018.04.18    sampling_2.8           htmlwidgets_1.3
## [58] httr_1.4.0            threejs_0.3.1         FNN_1.1.2.2
## [61] RColorBrewer_1.1-2    fpc_2.1-11.1          modeltools_0.2-22
```

```
## [64] pkgconfig_2.0.2      flexmix_2.3-14      nnet_7.3-12
## [67] caret_6.0-81         labeling_0.3        tidysselect_0.2.5
## [70] rlang_0.3.1          reshape2_1.4.3      later_0.7.5
## [73] munsell_0.5.0         mlbench_2.1-1       tools_3.5.2
## [76] LaplacesDemon_16.1.1 generics_0.0.2      RSQLite_2.1.1
## [79] pls_2.7-0            evaluate_0.12       stringr_1.3.1
## [82] mzID_1.20.1          yaml_2.2.0          ModelMetrics_1.2.2
## [85] knitr_1.21           bit64_0.9-7         robustbase_0.93-3
## [88] randomForest_4.6-14  purrr_0.3.0         dendextend_1.9.0
## [91] bindrcpp_0.2.2       ncdf4_1.16          nlme_3.1-137
## [94] whisker_0.3-2        mime_0.6            biomaRt_2.38.0
## [97] compiler_3.5.2       rstudioapi_0.9.0    e1071_1.7-0.1
## [100] affyio_1.52.0        tibble_2.0.1        stringi_1.2.4
## [103] highr_0.7            lattice_0.20-38     trimcluster_0.1-2.1
## [106] Matrix_1.2-15        gbm_2.1.5           pillar_1.3.1
## [109] BiocManager_1.30.4   MALDIquant_1.18     data.table_1.12.0
## [112] bitops_1.0-6         httpuv_1.4.5.1      R6_2.3.0
## [115] pcaMethods_1.74.0    affy_1.60.0         hwriter_1.3.2
## [118] bookdown_0.9         promises_1.0.1      gridExtra_2.3
## [121] codetools_0.2-15     MASS_7.3-51.1       gtools_3.8.1
## [124] assertthat_0.2.0     withr_2.1.2         diptest_0.75-7
## [127] hms_0.4.2           timeDate_3043.102   grid_3.5.2
## [130] rpart_4.1-13         class_7.3-14        rmarkdown_1.11
## [133] segmented_0.5-3.0    lubridate_1.7.4     shiny_1.2.0
## [136] base64enc_0.1-3
```

The source of this document, including the code necessary to reproduce the analyses and figures is available in a public manuscript repository on GitHub (O. Crook and Gatto 2019).

Reference

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