Image analysis for gene expression based phenotype characterization in yeast cells

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Role of 14-3-3 proteins and Nha1 antiporter in the response of *S.cerevisiae* to salt stress

> *In Chapter 2, we described our developed platform, i.e. YeastAnalysis. In this chapter, the developed platform is tested in studies on the role of* 14-3-3 *proteins and the* Nha1 $Na^+(K^+)/H^+$ *antiporter in the response of S. cerevisiae cells to high external* NaCl *concentrations. To this end we investigate the effect of high external* Na^+ *concentration on the levels of GFP-tagged* Bmh1, Bmh2 *and* Nha1. *For validation of the software tool the results were compared to results obtained by flow cytometry.*

This chapter is based on the following publication:

- Mohamed Tleis, Paul van Heusden and Fons J. Verbeek.*YeastAnalysis*: An image analysis platform to quantify fluorescent reporter proteins in *Saccharomyces cerevisiae* cells. (Submitted).

5.1 Introduction

Sacharomyces cerevisiae yeast as a model system is very suitable to study the function of proteins. This can be achieved through biochemistry and/or molecular biology or, if location is important through imaging. There is a class of proteins, referred to as the 14-3-3 proteins that are very important to maintain the integrity of the organism. These 14-3-3 proteins are found in all organisms and are often related to stress situations, i.e. disease [Rob02, Mar04]. Therefore, an experiment on stress in *S. cerevisiae* yeast is designed to further investigate these proteins. There we use salt stress, which can simply be induced by increasing the salt concentration in the medium.

S. cerevisiae has two genes encoding 14-3-3 proteins, *BMH1* and *BMH2*. In this study we address the function of the Bmh1 and Bmh2 14-3-3 proteins and the Nha1 cation transporter protein that modulates ion homeostasis. To address this function in the tolerance of yeast cells under salt stress, we cultivated strains expressing Nha1-GFP, Bmh1-GFP and Bmh2-GFP reporters under standard conditions and in the presence of 0.5 M NaCl. These reporter proteins are expressed by the *NHA1*, *BMH1* and *BMH2* genes tagged with the *GFP* reporter gene that expresses a green fluorescent protein. We also included a mutant strain with a deletion of *BMH1* gene (*Δbmh1*) expressing Nha1-GFP protein. Images were made by confocal laser scanning microscopy. As shown in Fig. 5.1, Nha1-GFP was mainly found in the plasma membrane, in agreement with the reported localization [Huh03]. Deletion of *BMH1* gene did not affect the localization of Nha1-GFP protein. Both Bmh1- and Bmh2-GFP were found all over the cell, also in agreement with previous reports [Huh03]. In order to fully understand the role of the 14-3-3 proteins and Nha1 protein in salt tolerance, the effect of NaCl on the levels of GFP-tagged proteins needs to be quantified. To this end we have used *YeastAnalysis* (cf. Chapter 2) to analyze microscope images using an extended set of features. In the following sections we discuss the materials and methods used in this experiment. Subsequently we discuss how the experiment analysis is performed including segmentation, measurement and data analysis. Then we discuss the results obtained from the application of *YeastAnalysis* to study the function of *BMH1*, *BMH2* and *NHA1* genes in the response to salt stress. We complete this chapter with a conclusion.

5.2 Materials and Methods

In this section we describe the materials and methods used to conduct the experiment explained in the introduction. The development of *YeastAnalysis* was already discussed in Section 2.8. Herein, we first highlight the used strains and how they were cultivated. Subsequently, we discuss the used microscopy and flow cytometry techniques.

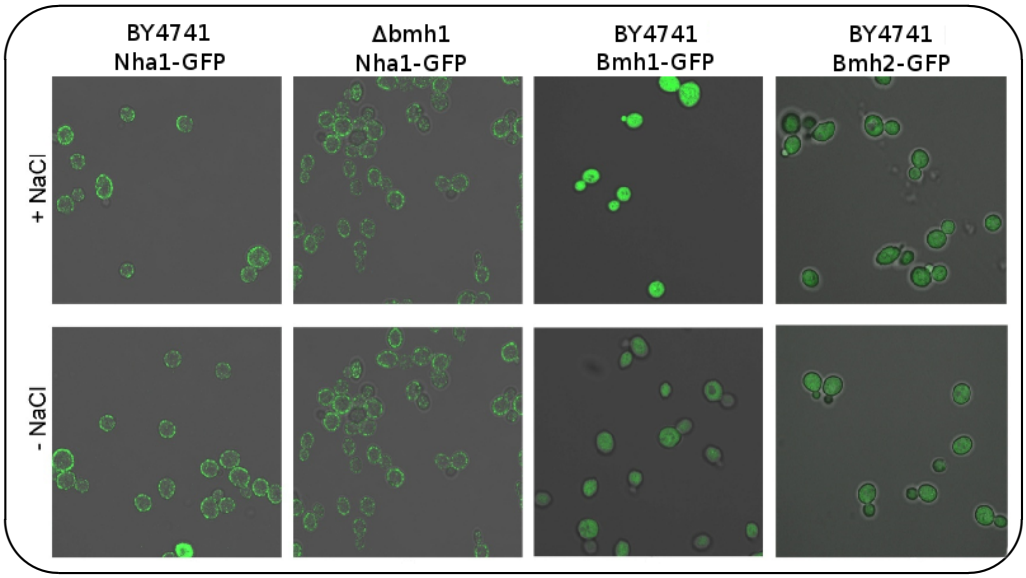

Figure 5.1: *Confocal laser scanning microscopy of* BY4741 NHA1-GFP, *Δbmh1* NHA1-GFP, BY4741 BMH1-GFP *and* BY4741 BMH2-GFP *cells grown in supplemented* MY *medium with (+*NaCl*) or without (−*NaCl*) additional* 0.5 M NaCl.

5.2.1 Yeast strains and cultivation

The yeast strains used in this study are listed in Table 5.1. *Δbmh1 NHA1-GFP* was constructed by integration of GFP downstream of the *NHA1* gene coding sequences in strain *Δbmh1* (GG3240). For this purpose a PCR fragment was generated using pYM28 [Jan04] as template and the primers pYM-NHA1-Fw (5′-GCTGCTGTTAAGTCGGCGCTATCAAAAACGCTT GGTCTCAATAAGCGTACGCTGCAGGTCGAC-3) and pYM-NHA1-Rev (5′-CGACACA TGTAAATAAAAAAGGCATTTCGTTTATATATATACTAAATCGATGAATTCGAGC TCG-3′). Transformants were selected for histidine prototrophy. Yeast transformations were performed using the LiAc method [Gie95]. Yeast was cultivated in MY medium supplemented, when required, with histidine, methionine, uracil and leucine [Zon86].

For microscopy and flow cytometry, yeast cells were grown overnight in MY medium supplemented with histidine, methionine, uracil and leucine, when required. The next morning cultures were diluted ten-fold in supplemented MY medium containing 0.5 M NaCl or in the same medium without added NaCl. After cultivation for 4 to 7 hours at 30°C, cells were analyzed by confocal microscopy and/or flow cytometry.

Strain	Genotype	Source/Reference
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf,Germany
Δbmh1 (GG3240)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bmh1Δ::loxP*	Wouter Hendriksen, unpublished results
NHA1 – GFP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 NHA1-GFP (HIS3MX6)*	Life Technologies
BMH1 – GFP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 BMH1-GFP (HIS3MX6)*	Life Technologies
BMH2 – GFP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 BMH2-GFP (HIS3MX6)*	Life Technologies
Δbmh1 NHA1-GFP (GG3398)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bmh1Δ::KAN.MX NHA1-GFP (HIS3MX6)*	This study

Table 5.1: *Yeast strains used in this study.*

5.2.2 Confocal microscopy and flow cytometry

For image acquisition a Zeiss LSM 5 Exciter-AxioImager M1 confocal microscope with a Plan-Apochromat objective (63X/1.4 Oil DIC) and Zeiss ZEN 2009 software were used. GFP was imaged with excitation at 488 nm and emission at 505-530 nm. For flow cytometry, a Merck-Millipore Guava EasyCyte 5 Flow Cytometer was used. Fluorescence was determined after excitation at 488 nm and using the standard green 525/30 nm emission filter. For each flow cytometry analysis, 5000 cells were used.

5.3 Experiment Analysis

Here we discuss the steps followed in the analysis of the experiment images; i.e. segmentation, measurement and data analysis.

5.3.1 Segmentation

The first step in the analysis is to locate the individual cells in the image through segmentation. In this experiment, we apply segmentation on the bright-field channel of the image, and we use the segmentation results to analyze the other channels. In the bright-field images, the cell contours are visible as dark structures. We applied the *HCSP* segmentation algorithm described in Section 3.2 and Section 3.4.2. It first applies a 3x3 *Prewitt* filter, which gives a good initial estimate of the magnitude of the gradient of the image. i.e. it highlights the cell contours. On this gradient image, a threshold and skeletonization algorithm [Gon08] are applied to get the contours as binary structures of one pixel thickness. The following step is the utilization of Hough Transform to locate the structures that form part of a geometrical circle, for the fact that

yeast cells are nearly circular. The detected partial circumference of this circle corresponds to the detected part of the cell contour, and the center point of this circle corresponds to a point somewhere in the middle of the cell. The location of the center point is not necessarily at the center of the cell, as the next step creates a sufficient resampled polar image to evaluate the complete cell. This subsequent step is the extraction of the exact contour of the cell starting from the center point for that cell located by Hough transform. Figure 5.2(a) shows an example of a cell after locating its center point by Hough Transform. The *red* circles and *blue* lines are labels to illustrate the creation of the resampled polar image of the cell as shown in Fig. 5.2(b). The radius at an angle θ (*blue* lines in Fig. 5.2(a)) in the image plane, is transformed into a column in the polar image (vertical blue lines in Fig. 5.2(b)). The circles surrounding the center point (*red* circles of radius r in the image plane in Fig. 5.2(a)), are transformed into rows of the polar image (red horizontal lines in Fig. 5.2(b)). Next, a minimal path algorithm is applied on the polar image to find the full contour of the cell. Figure 5.2(c) shows the detected minimal path in *cyan*. Figure 5.2(d) shows the detected cell contour as *cyan* pixels backprojected to the original image. The results of segmentation of a typical microscope image are shown in Fig. 5.3.

During the segmentation process, outliers are discarded by setting limits to the minimum and maximum cell size as well as to the minimum circularity of the detected shape. The extracted cell contours or the masks are subsequently used to measure the fluorescence intensity in the overlaid fluorescence channel, and for measurements in the bright-field channel.

5.3.2 Measurement

After detection of the cells in the microscope images, the *YeastAnalysis* software is used to perform various measurements on these cells. In addition to the fluorescence intensity, several shape features of the cells are measured in the bright-field image, including size (area), perimeter, density, and features describing the textures of the cell such as variance, relative smoothness, skewness, uniformity and entropy [Gon08]. The same set of features are measured in the bright-field channel as well as in the overlaid fluorescence image channels. Table 5.2 lists how texture measurements can possibly be interpreted within images of *S. cerevisiae* cell.

For analysis of membrane proteins *YeastAnalysis* automatically estimated the region where the cell membrane protein are expressed by focusing on the pixels next to the cell contour (Fig. 5.4). All measurements are saved automatically into a CSV (comma-separated values) file as explained in Section 2.4. This measurement file is used at the following step to generate a report and for further inspection by a specialist.

5.3.3 Data Analysis

After completion of the measurements, two sets of cells are compared to study any differences between those sets. The first set is those cells cultivated in a *non*-NaCl medium, while the second set is those cultivated in a 0.5 M NaCl medium. *YeastAnalysis* automatically generates a report in *pdf* format holding basic statistics and graph charts to visualize the results.

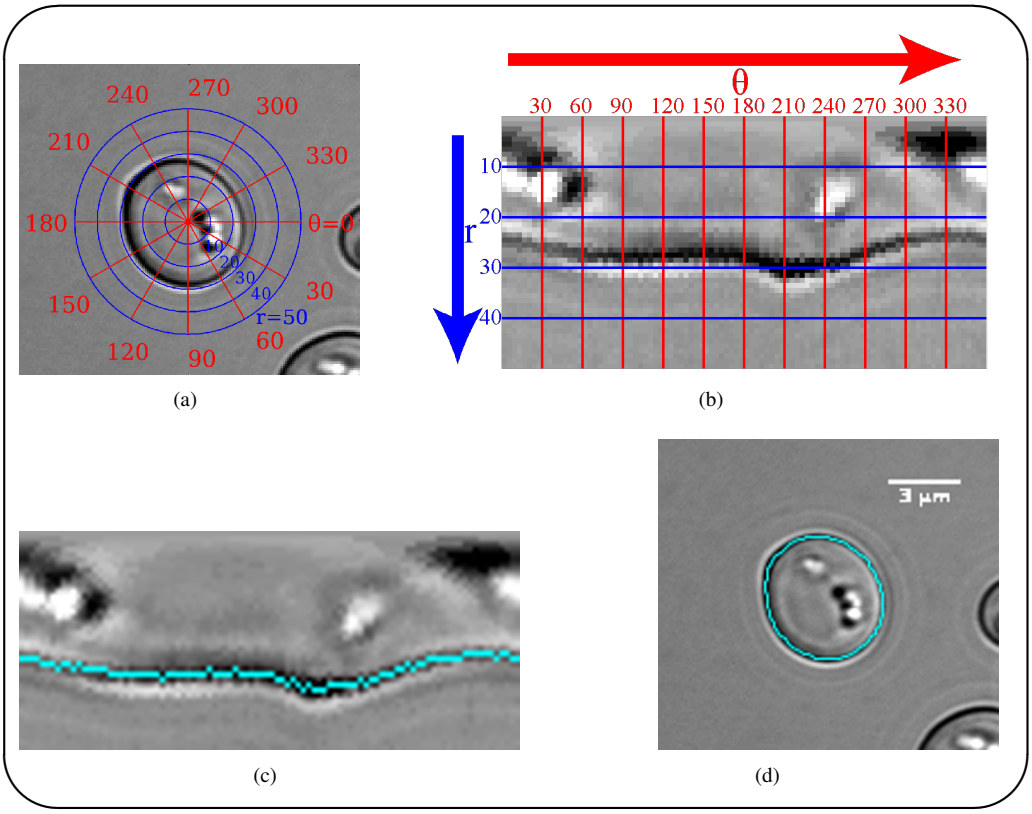

Figure 5.2: ***Detecting contours by Hough Transform and Minimal Path algorithm.*** *Image (a) shows an example of a cell after detection of the center point by Hough Transform. In (b) a polar image is generated. The columns correspond to the pixels along the radius at an angle θ of the largest possible circle (red lines). The rows correspond to the circles surrounding the center point (blue lines, blue circles in image a). Image (c) shows the detected path from the first column to the last column in the polar image, after the application of dynamic programming. Image (d) shows the detected path as the actual contour of the cell.*

The statistical information includes the number of detected cells and the mean values of the different measured features along with their standard deviations. The unpaired Student t-test is performed to report the t-value and p-value to assist in analyzing the significance of the differences between two sets of cells. To visualize the measurement results, several chart types are generated, including scatter plots, Pareto charts and box-and-whiskers plots. The next section shows the results obtained using *YeastAnalysis* to study the function of *BMH1, BMH2,* and *NHA1* genes under salt stress.

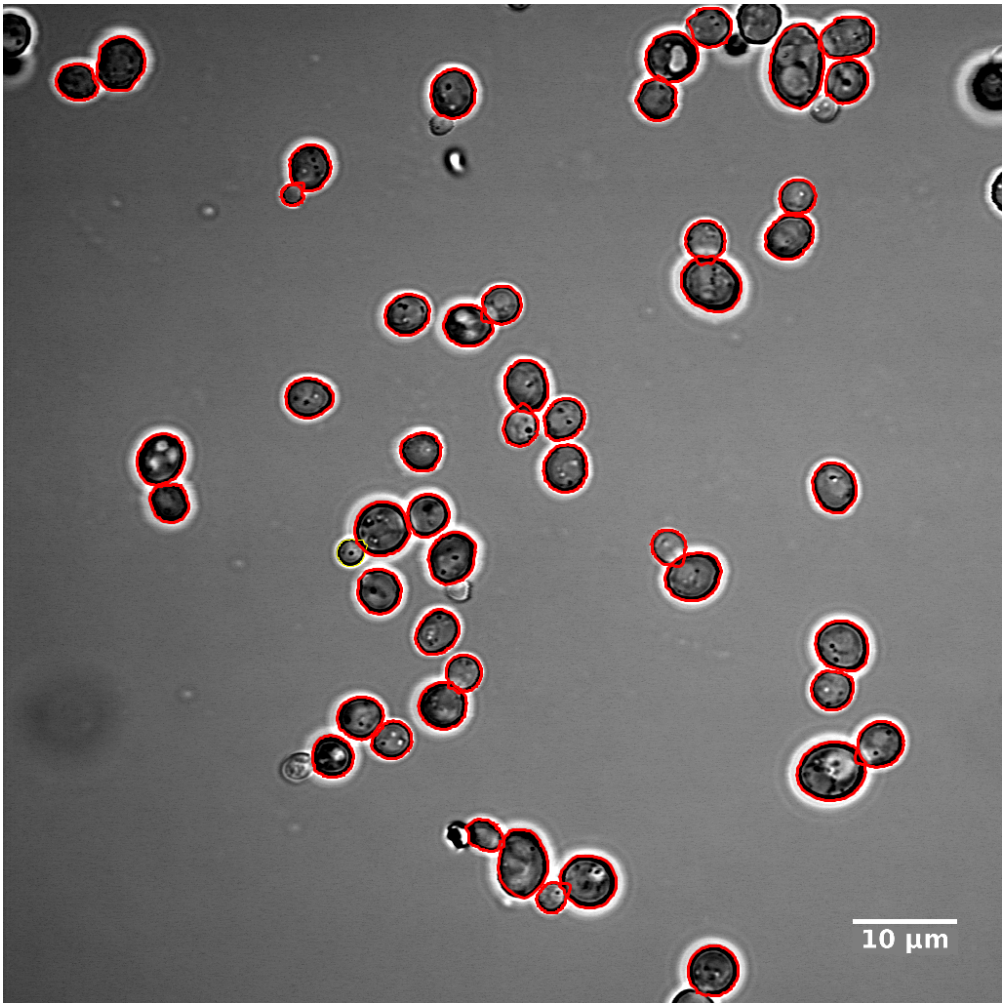

Figure 5.3: *Overlay on a bright-field image showing the extracted contours of the detected cells.*

Texture	Bright-Field Channel	Fluorescent Channel
Entropy	A higher entropy value can indicate a distorted cell as in cell death.	Lower entropy values can indicate that expressed proteins are forming simple patterns within cells. Higher values can indicate a more complex and variable patterns.
Skewness	Lower skewness values can indicate brighter cells while higher values can indicate darker cells.	Lower Skewness values can indicate less intensity while higher values can indicate higher intensity values within the cell.
Uniformity	Uniformity values go lower when there are no dark structures or organelles appearing within the cell, or when the cell is not distorted.	Uniformity values can go higher when the gene is expressed more evenly throughout the cell.
Variance	Indicates how many dark structures appear or how distorted a cell is	Indicates a constant expression throughout the cell (low variance), or an highly variable expression throughout the cell (high variance).

Table 5.2: *Interpretation of texture measurements in yeast.*

5.4 Results

We validated *YeastAnalysis* in studies on the effect of salt stress on yeast cells expressing GFP-tagged Bmh1, Bmh2 or Nha1. To this end, six to ten confocal microscope images were acquired with in total more than 200 cells for each condition. We have four different conditions in two different classes; i.e. the *non*-NaCl and the 0.5 M NaCl class. The number of samples per condition per class are depicted in Table 5.3. The acquired images in this experiment contain two channels, a fluorescence channel and a bright-field channel. The fluorescence images show the expression of *BMH1-GFP, BMH2-GFP, NHA1-GFP* and *Δbmh1 NHA1-GFP* in yeast cells cultured in the absence or presence of additional 0.5 M NaCl. We loaded these images into *YeastAnalysis* and used the *HCSP* algorithm for detection of the cells in the bright-field image. By automatic segmentation of the total acquired 195 microscope images in this study we were able to detect 92 percent of the cells on these images, whereas 2.2 percent of the detected objects did not correspond to learned shape of a healthy cell; i.e. debris and dead cells. The F1-Score [Rij79] is used to measure the effectiveness of the algorithm. The F1-Score measure is 0.95. The missing cells were added by manual segmentation and the false positives were removed. Such manual segmentation is easily performed as non-detected cells are added

through manual seeding and incorrect detections are deleted. These adjustments are facilitated through an interactive Graphical User Interface (GUI) in the *YeastAnalysis* software.

The segmented images were used for measurement of various cell features. These measurements are exported into a csv-file showing the cells expressing GFP-tagged Bmh1, Bmh2 and Nha1 cultured in the absence and presence of additional 0.5 M NaCl. For the measurements of the fluorescence channel, we selected the most common features; i.e. the total intensity, cell size, cell perimeter, internal density, intensity per pixel, intensity per square micron, circularity, variance, skewness, uniformity, relative smoothness and entropy.

The results of the measurements of the cell size and fluorescence intensity in a typical experiment on the effect of additional 0.5 NaCl on cells expressing Nha1-GFP, Bmh1-GFP

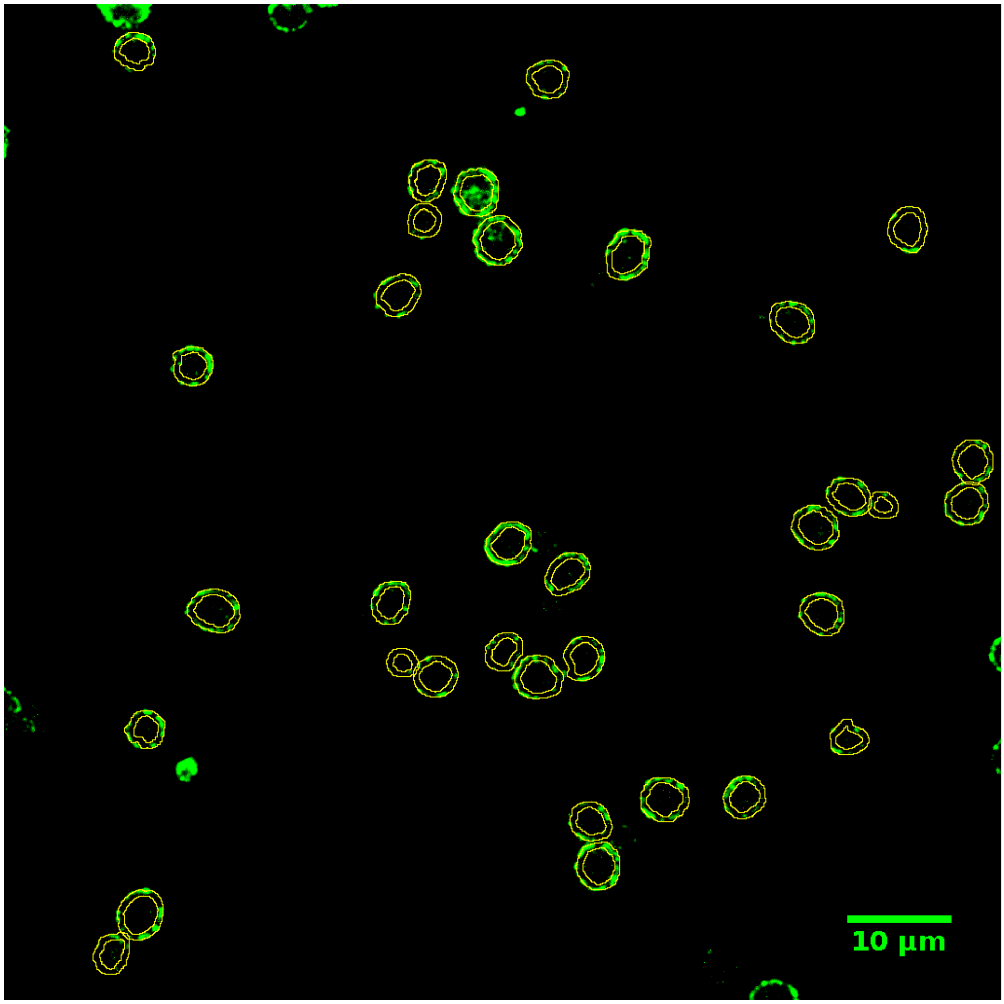

Figure 5.4: *Automatically generated overlay showing estimated cell membrane locations.*

Strain	Cell Size in μm^2		Fold Change Cell Size +NaCl/ -NaCl (P-Value)	Integrated GFP fluorescence		Fold Change GFP +NaCl/ -NaCl (P-Value)
	-NaCl	+NaCl		-NaCl	+NaCl	
NHA1-GFP	16.7±5.2 (407)	15.6±4.7 (281)	0.93 P = 0.002	4.3±2.2 (407)	4.5±2.6 (281)	1.05 P = 0.44
Δbmh1 NHA1-GFP	13.6±4.2 (915)	15.3±5.6 (219)	1.12 P < 0.001	3.2±1.7 (915)	5.6±2.9 (219)	1.75 P < 0.001
BMH1-GFP	12.7±4.2 (447)	12.6±4.5 (328)	0.99 P = 0.74	32.6±15.2 (447)	56.6±18.3 (328)	1.74 P < 0.001
BMH2-GFP	12.9±4.5 (311)	11.7±3.7 (238)	0.91 P = 0.001	20.0±10.4 (311)	33.9±13.8 (238)	1.70 P < 0.001

Table 5.3: *Determination of cell size and GFP fluorescence in microscope images using YeastAnalysis. The statistical method used to check the significance of the differences (P-Value) was the Student t-test. Numbers within parenthesis represents the number of cells used in the analysis. Arbitrary unit is used for GFP fluorescent.*

Strain	GFP fluorescence [Arbitrary Units]		Fold Change GFP fluorescence +NaCl/-NaCl
	-NaCl	+NaCl	
NHA1-GFP	2.53	2.98	1.18
Δbmh1 NHA1-GFP	1.83	2.72	1.49
BMH1-GFP	77.3	131.0	1.69
BMH2-GFP	43.5	65.6	1.51

Table 5.4: *Determination of GFP fluorescence using flow cytometry.*

or Bmh2-GFP are summarized in Table 5.3. Moreover, the effect of deletion of *BMH1* gene on Nha1-GFP expression is shown. The results show that NaCl stress results in a slight increase in the expression of Nha1-GFP in the wild type BY4741 background but a stronger increase in the mutant *Δbmh1* background, whereas the expression of both Bmh1- and Bmh2-GFP is strongly increased upon salt stress. The results further show that *Δbmh1* cells are slightly smaller than wild type BY4741 cells (P-values from *Student* t-test less than 0.001 as seen in Table 5.3). Figure 5.5 shows some visualization charts generated by *YeastAnalysis* for this analysis of *Δbmh1 NHA1-GFP* strain under salt stress. Figure 5.6 shows the analysis of their membrane intensity and size. All the experiments were repeated three times except for the cells expressing Bmh2-GFP that was repeated in duplo and similar results were obtained.

To validate the results obtained by analyzing microscope images by *YeastAnalysis* we used flow cytometry. To this end 5000 cells from each culture were analyzed and the *GFP*-fluorescence measurements are shown in Fig. 5.7. Calculation of the average GFP fluorescence

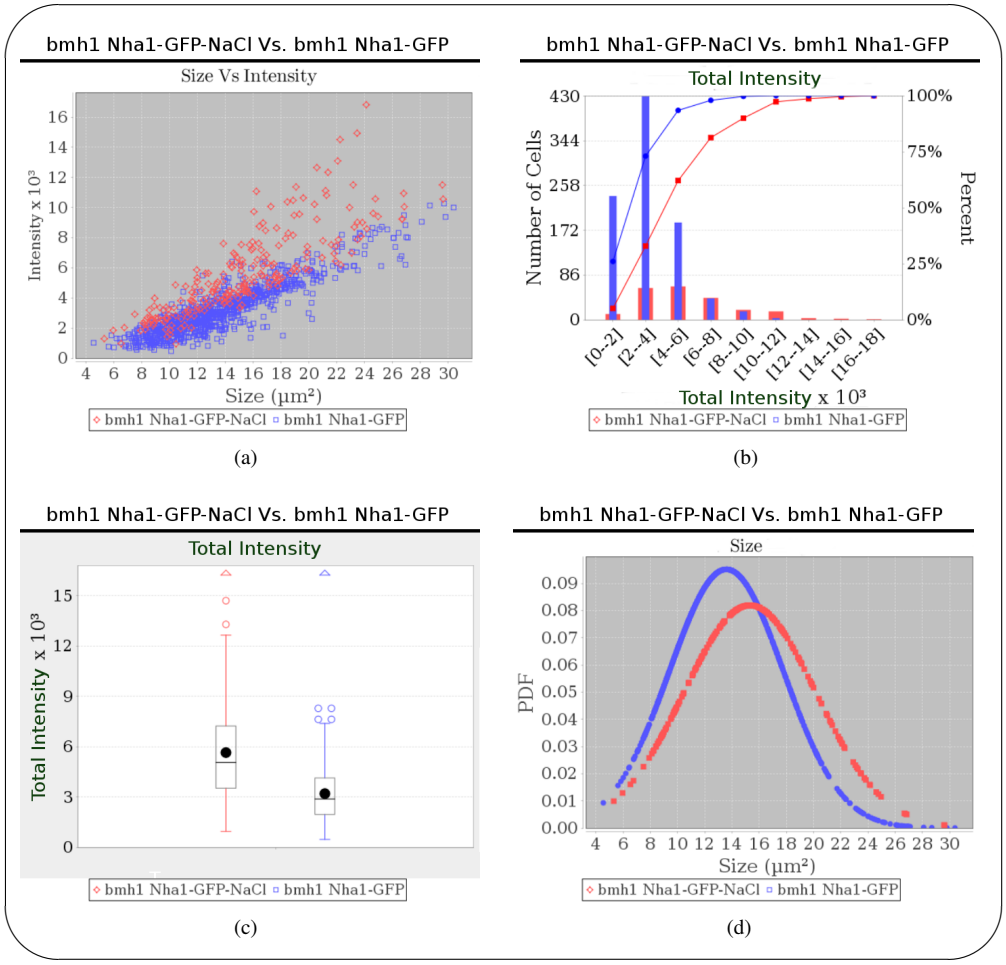

Figure 5.5: ***Visualization of data generated by YeastAnalysis.*** *Yeast strain Δbmh1 NHA1-GFP was cultivated in* MY *medium supplemented or not supplemented with* 0.5 M NaCl *and cells were analyzed by microscopy, followed by analysis of the images using YeastAnalysis. Results can be visualized in several ways. (a) - in Scatter Plot showing the cell size on the x-axis and fluorescent Intensity on y-axis. (b) - in a Pareto chart of the fluorescence intensity vs the number of cells. (c) - in a Box and Whiskers Plot. (c) - in a Scatter Plot fitted to Gaussian Distribution showing the cell size on the x-axis and the Probability Density Function (PDF) on the y-axis. Statistical analysis revealed that* bmh1 Nha1-GFP *total fluorescence intensity increased significantly upon salt stress (*1.75*-fold; P<* 0.001*). The size is increased significantly upon salt stress as well (*1.12*-fold; P<* 0.001*).*

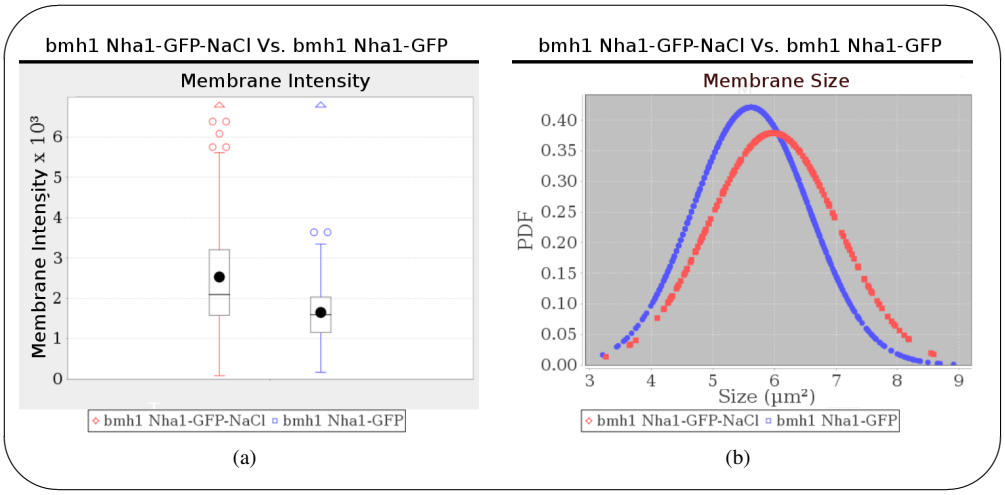

Figure 5.6: ***Membrane Intensity of Δbmh1 NHA1-GFP cell strain.*** *Statistical analysis revealed that bmh1* Nha1-GFP *fluorescence membrane intensity increased significantly upon salt stress (*1.53*-fold; P<* 0.001*). The size that this membrane protein occupies constitutes of an average of* 40 *percent of the total cell size.*

showed that the expression of Nha1- Bmh1- and Bmh2-GFP increased upon NaCl stress (Table 5.4). In summary, the results obtained by confocal microscopy and image analysis using *YeastAnalysis* and the results obtained by flow cytometry are in very good agreement.

YeastAnalysis is able to measure GFP intensity in the membrane (Fig. 5.4). In a typical experiment we showed that 63 percent of the GFP fluorescence was found around the membrane in BY4741 NHA1-GFP cells, whereas the membrane protein region constitutes, in average, 40 percent of the cell area (cf. Fig. 5.6). This result indicates that the majority of the Nha1 protein is expressed nearby the membrane, which corresponds well with the reported localization [Huh03].

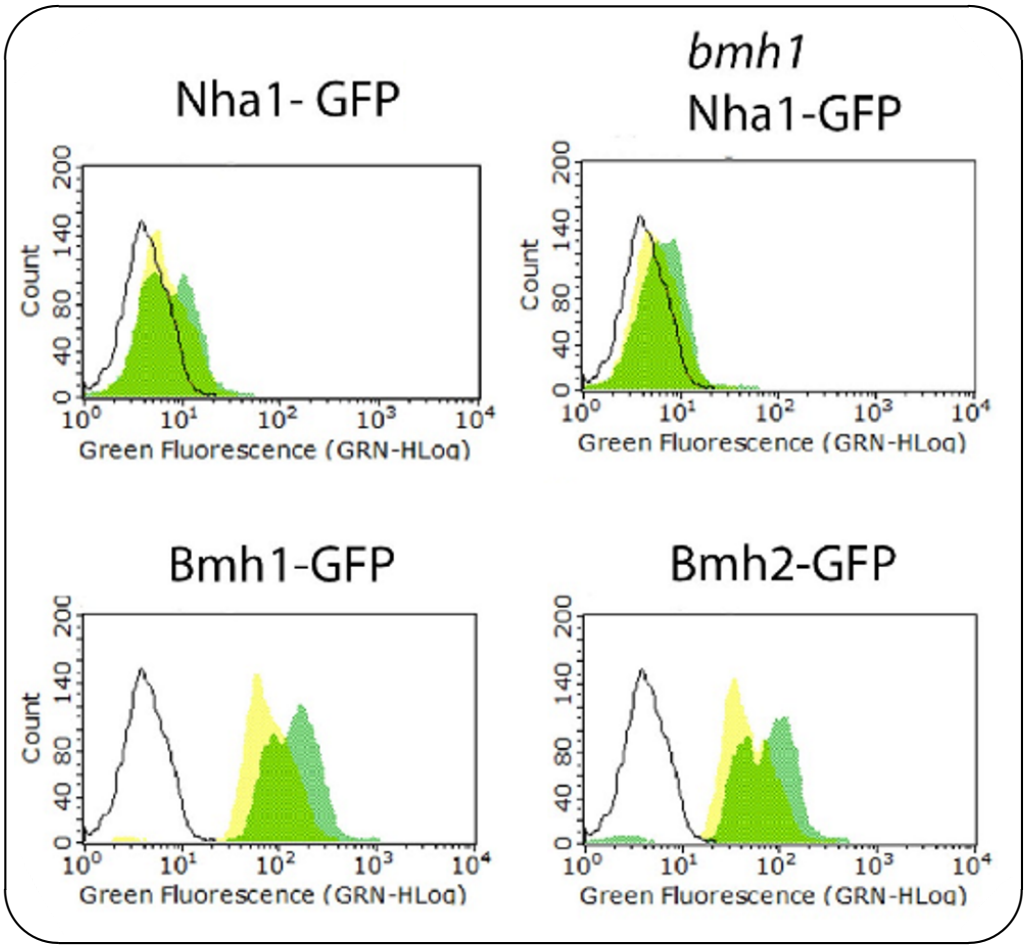

Figure 5.7: ***Flow cytometric analysis of the effect of*** 0.5 M NaCl ***on yeast cells expressing*** GFP***-tagged proteins.*** NHA1-GFP, BY4741 *expressing* Nha1-GFP*; bmh1* Nha1-GFP, *Δbmh1, expressing* Nha1-GFP*;* Bmh1-GFP, BY4741 *expressing* Bmh1-GFP*;* Bmh2-GFP, BY4741 *expressing* Bmh2-GFP. *Yellow: cells cultivated in the absence of additional* NaCl*; green: cells cultivated in the presence of* 0.5 M NaCl*; solid line, no fill:* BY4741 *cells.*

5.4.1 Yeast Vacuoles

Using the *YeastAnalysis* software, the size and number of vacuoles for an individual cell can be estimated. As some gene expression causes a fluorescence all over the cytoplasm, this fact can be used to find the size and shape of the vacuoles. In this experiment we study the vacuole of cells from *BMH1-GFP* wildtype strains. Our objective is to study the vacuole size and number of vacuolar compartments under salt stress in both strains. First we analyze the size of the central vacuoles. Subsequently we analyze the number of vacuolar compartments and their total size. After that, we repeat the experiments using images acquired from different experiments at different time points.

In our dataset we have 42 segmented cells from *BMH1-GFP* strain cultivated in 0.5 M NaCl, 131 *BMH1-GFP* cultivated under non-NaCl medium. In our analysis we compute the relative vacuole size; i.e. the vacuole size in terms of percentage of the cell size. In the first analysis we noticed that the central vacuole size occupies in average 3.5% of the cell size in *BMH1-GFP* strain, increased to 5.3% in 0.5 M NaCl. This result is depicted in Fig. 5.8 and Table 5.5(a). In order to assess the significance of this increase, we perform an unpaired Student t-test of the null hypothesis such that the means of the two samples (i.e. under non-NaCl and that of 0.5 M NaCl) are equal. The p-value from the *Student* t-test statistical analysis is 0.021 as shown in Table 5.5(a).

The mean value does not show clear significant difference in the first analysis. Therefore we perform additional analysis in which outliers are removed. From our observations of yeast images we have observed outlier cases such as very few large cells that might bias the measurement and cells with very high intensity values due to auto-fluorescence from a dead

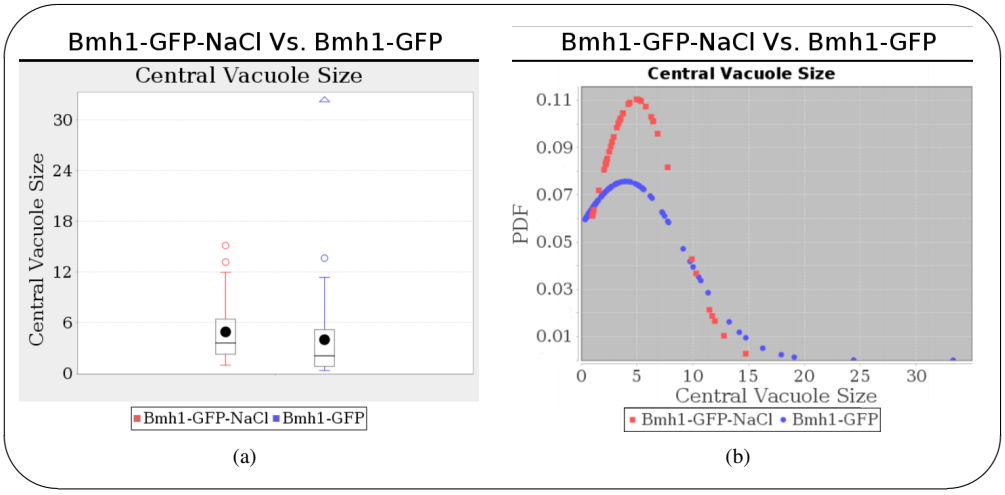

Figure 5.8: ***Central vacuole estimation in BMH1-GFP cells under salt stress.*** *(a) - Box and whiskers chart comparing BMH1-GFP under non-*NaCl *and* 0.5 M NaCl *medium. (b) - Fitting measurement in (a) to a Gaussian distribution.*

cell. Deviation from the mean is widely used to set outlier threshold, however, significant results could easily turnout to be false positives. In literature [Ley13], deviation from the median is proposed, as the median is very insensitive to the presence of outliers. The Median Absolute Deviation (*MAD*) is used as a way of dealing with the problem of outliers. To define a rejection criterion, we have adopted the recommended coefficient value of 2.5 [Ley13]. The recommended threshold value for outlier detection is at the median plus or minus 2.5 times the *MAD*. By removing these outliers, difference between the mean vacuole size in the two groups becomes more significant. This result is shown in Fig. 5.9 and Table 5.5(a). The estimated average vacuole size is increased from 2.3% to 3.6% under stress. The p-value from *Student* t-test is 0.002. Nevertheless, this analysis suggests that the central vacuole has a larger volume under salt stress. This is an interesting result, but what about all the vacuoles within the cells? Hence, we perform an additional experiment considering all estimated vacuoles.

In addition to the central vacuole we analyzed the number of vacuolar components and their total size per individual cells. Similarly to the central vacuole analysis, we compute the relative vacuole size; i.e. the vacuole size in terms of percentage of the cell size. Figure 5.10 illustrates typical yeast cells expressing Bmh1-GFP, where the central vacuole and all other vacuoles are segmented.

In this analysis we noticed interestingly, as it is clear from Fig. 5.11 that the number of vacuolar compartments significantly increased under NaCl salt stress in the *BMH1-GFP* strain. This increase is from an average of two vacuolar compartments under non-NaCl medium to five compartments under 0.5 M NaCl.

From Fig. 5.12 and Table 5.5(b), we can see that the total vacuoles size occupies in average 4.6% of the cell size in *BMH1-GFP* strain, increased to 8.7% in 0.5 M NaCl. The p-value from the *Student* t-test statistical analysis is less than 0.001 as shown in Table 5.5(b).

We performed an additional analysis by removing outliers as done with the analysis of central vacuoles. The result is shown in Fig. 5.13 and Table 5.5(b). The estimated average vacuoles size is increased from 3.1% to 7.0% under stress. The p-value from *Student* t-test

(a)

	-NaCl	+NaCl	P-value
Initial Analysis	3.5% ± 5.1 (131)	5.3% ± 4.1 (42)	0.021
No-Outliers	2.3 %± 2.0 (94)	3.6% ± 1.9 (34)	0.002

(b)

	-NaCl	+NaCl	P-value
Initial Analysis	4.6% ± 6.0 (131)	8.7% ± 4.9 (42)	< 0.001
No-Outliers	3.1% ± 2.5 (84)	7.0% ± 3.1 (34)	< 0.001

Table 5.5: *Analysis of vacuole size in* Bmh1 14-3-3 *protein under non-*NaCl *stress and* 0.5 M NaCl *stress level. The values represent the mean and standard deviation of the relative percentage of vacuole size related to the cell size. The p-value is computed from the Student t-test statistical analysis. The numbers in parentheses represent the number of cells in each condition. (a) - Descriptive analysis of central vacuole sizes before and after removal of outliers. (b) - Descriptive analysis of total vacuole sizes before and after removal of outliers.*

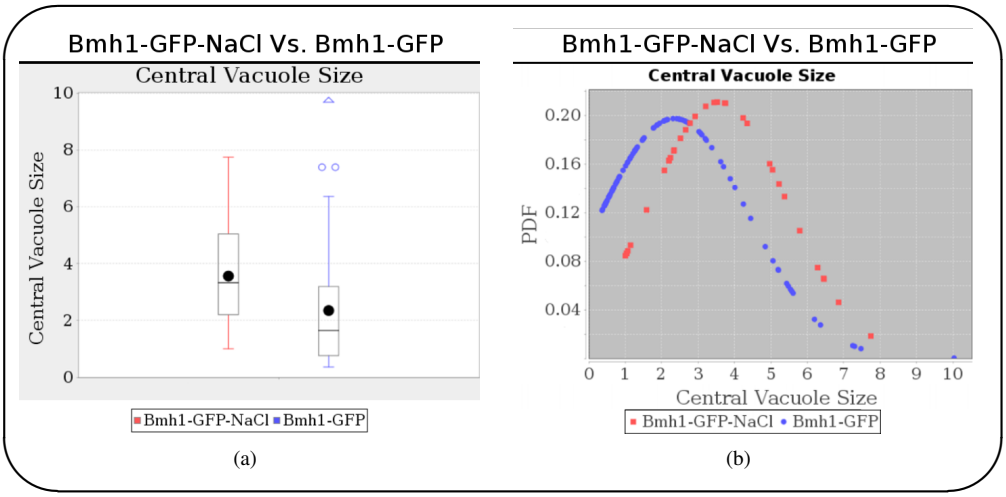

Figure 5.9: ***Central Vacuole estimation in BMH1-GFP under salt stress and after outliers removal.*** *(a) - Box and whiskers chart comparing BMH1-GFP under non-*NaCl *and* 0.5 M NaCl *medium. (b) - Fitting measurement in (a) to a Gaussian distribution.*

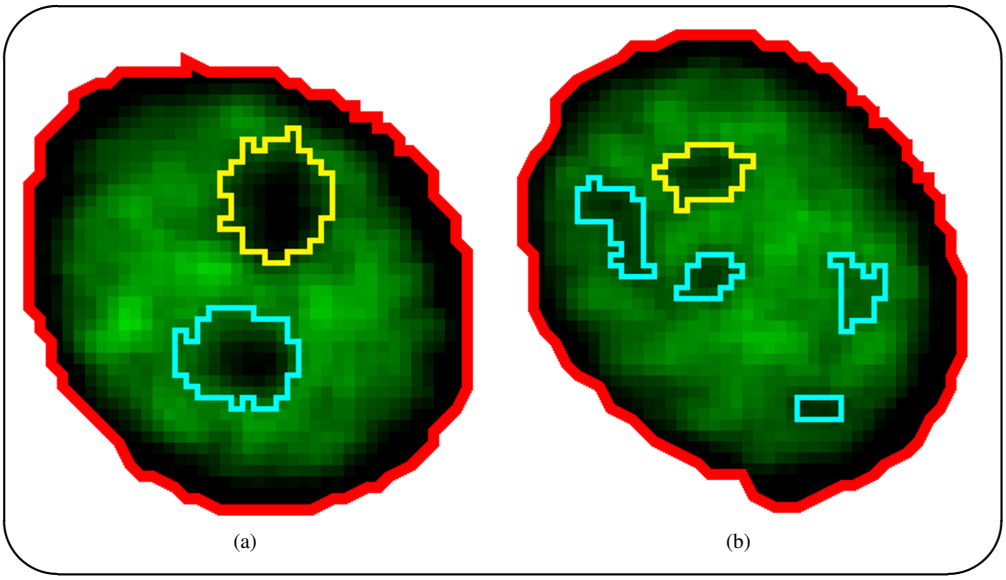

Figure 5.10: ***Estimation of vacuoles in sample yeast cells expressing*** Bmh1-GFP ***protein.*** *The detected central vacuole is marked with a yellow contour and all the other vacuoles are marked with cyan countors. (a) - Sample yeast cell with two vacuoles. (b) - A sample cell with one large central vacuole and multiple small vacuoles.*

is again less than 0.001. Nevertheless, this analysis suggests that also the total vacuoles has a larger volume under salt stress as well as the central vacuole. The result in this analysis is interesting. However, it requires more experiment validation. The increase in the number of

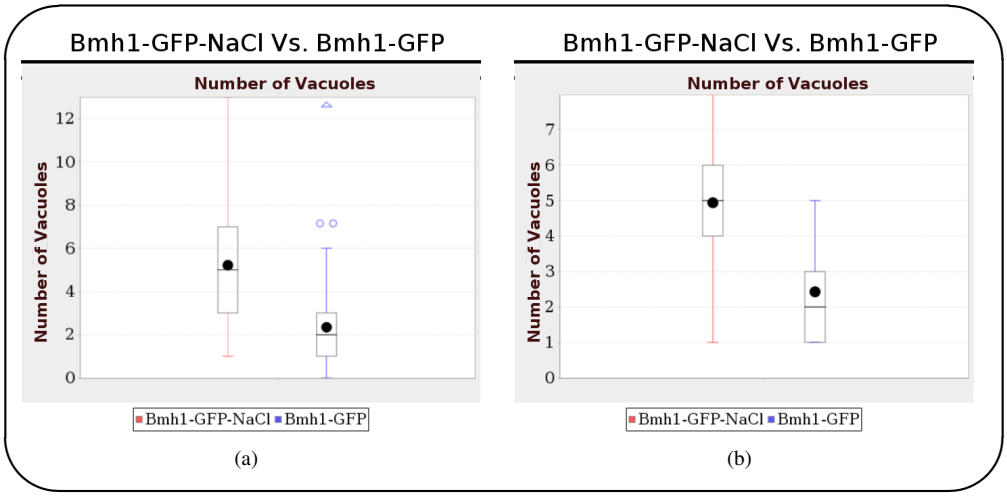

Figure 5.11: ***Number of Vacuolar Compartments in cells expressing* Bmh1** 14-3-3 ***proteins under salt stress.*** *(a) - Number of vacuoles in BMH1-GFP under non-*NaCl *and* 0.5 M NaCl *medium. (b) - Number of vacuoles in BMH1-GFP after outliers removal.*

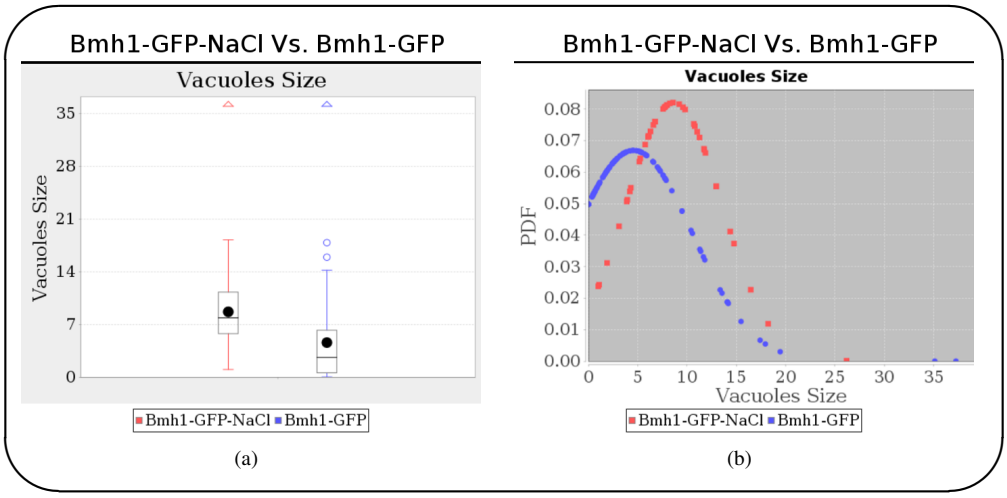

Figure 5.12: ***Total vacuole sizes estimation in BMH1-GFP cells under salt stress.*** *(a) - Box and whiskers chart comparing BMH1-GFP under non-*NaCl *and* 0.5 M NaCl *medium. (b) - Fitting measurement in (a) to a Gaussian distribution.*

vacuolar compartments might be a mechanism for water conservation in yeast cells. These small provacuoles in cells under salt treatment could have appeared as a result of dehydration. This result is consistent with another study done with plant root cells [Sán92]. However, the increase in total vacuole size and central vacuole size is opposite to what we expected. Because we expected the cellular volume to decrease in response to increase in external salinity. Hence we analyzed images acquired in three other experiments performed at different dates. Interestingly, in all the experiments the estimation of vacuoles size has increased under NaCl stress for *BMH1-GFP* strain with p-values from *Student t-test* being < 0.001, 0.03 and 0.3 with a fold change of 1.39, 1.19 and 1.12 respectively. The result of the repeated experiments for *BMH1-GFP* cells are depicted in Table 5.6. After outliers removal these numbers are shown in Table 5.7. The number of vacuoles seems to be always increasing with *BMH1-GFP* under NaCl stress, although not significantly in one experiment.

These results are unexpected. In order to validate if these results are correct, we propose to perform an experiment in which we stain the vacuoles with a bio-marker. From the literature [Rob91], there are different protocols available for the staining of the vacuoles. This experiment will help us to better understand the behaviour of cells under salt stress, and whether the number of vacuolar components and vacuole sizes increase as a mechanism that the cell adopt under stress. The stained vacuoles can be easily measured in *YeastAnalysis*, which has an option to measure separate channels of any stain/fluorescent label.

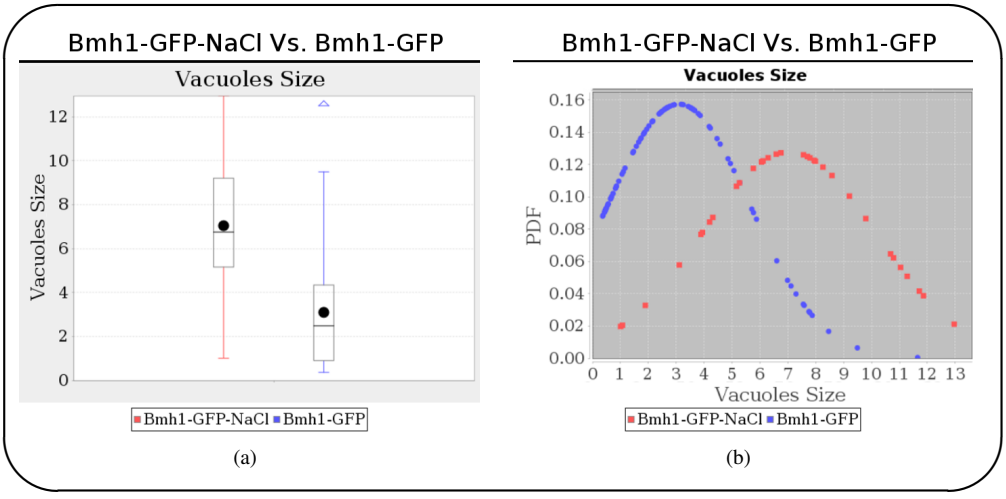

Figure 5.13: ***Total vacuole sizes estimation in BMH1-GFP cells under salt stress after outliers removal.*** *(a) - Box and whiskers chart comparing BMH1-GFP under non-*NaCl *and* 0.5 M NaCl *medium. (b) - Fitting measurement in (a) to a Gaussian distribution.*

Repetitions BMH1-GFP	Vacuoles Size		Size Fold Change +Nacl/-Nacl (P-Value)	Number Of Vacuolar Compartments		Vacuoles Fold Change +Nacl/-Nacl (P-Value)
	-NaCl	+NaCl		-NaCl	+NaCl	
Experiment 1	10.0 ± 4.4 (166)	13.9 ± 8.2 (155)	1.39 (P<0.001)	7.2 ± 4.0 (166)	8.1 ± 4.6 (155)	1.16 (P : 0.068)
Experiment 2	9.1 ± 5.1 (88)	10.8 ± 4.2 (86)	1.19 (P: 0.035)	5.4 ± 3.2 (88)	5.6 ± 2.7 (86)	1.04 (P : 0.717)
Experiment 3	6.7 ± 4.6 (74)	7.5 ± 4.0 (57)	1.12 (P: 0.324)	5.0 ± 3.8 (74)	5.3 ± 2.9 (57)	1.06 (P : 0.611)

Table 5.6: *Repeated experiments to determine vacuoles size and number of vacuolar compartments. The statistical method used to check the significant of the differences (P-Value) was the Student t-test. Numbers within parenthesis represents the number of cells used in the analysis.*

Repetitions BMH1-GFP	Vacuoles Size		Size Fold Change +Nacl/-Nacl (P-Value)	Number Of Vacuolar Compartments		Vacuoles Fold Change +Nacl/-Nacl (P-Value)
	-NaCl	+NaCl		-NaCl	+NaCl	
Experiment 1	9.7 ± 3.5 (139)	11.7 ± 4.5 (130)	1.21 (P<0.001)	6.6 ± 2.9 (139)	7.7 ± 3.8 (130)	1.17 (P : 0.008)
Experiment 2	7.7 ± 3.4 (70)	9.4 ± 3.7 (72)	1.22 (P:0.006)	5.0 ± 2.2 (70)	5.7 ± 2.6 (72)	1.14 (P : 0.075)
Experiment 3	6.1 ± 3.8 (58)	6.5 ± 2.8 (49)	1.07 (P: 0.585)	4.3 ± 2.6 (58)	5.0 ± 2.7 (49)	1.16 (P : 0.206)

Table 5.7: *After removal of outliers from the repeated experiments to determine vacuoles size and number of vacuolar compartments. The statistical method used to check the significant of the differences (P-Value) was the Student t-test. Numbers within parenthesis represents the number of cells used in the analysis.*

5.5 Conclusion

We have used *YeastAnalysis* to address the role of 14-3-3 proteins and the Nha1 cation transporter in the response of yeast cells to high salt concentrations. 14-3-3 proteins are highly conserved eukaryotic proteins binding to hundreds of different mostly phosphorylated proteins [for reviews see: [Mac04, Ait06, Mor09]]. In addition, the *S. cerevisiae* 14-3-3 proteins, encoded by *BMH1* and *BMH2*, bind to hundreds of phosphorylated proteins [Heu95, Kak07, Heu09] and play a role in the regulation of many processes including tolerance to NaCl [Pos00, Zah12]. Deletion of *BMH1*, encoding the major 14-3-3 isoform, is known to result in an increased sensitivity to Na^+, Li^+ and K^+ and to cationic drugs [Zah12]. Testing the genetic interaction between *BMH* genes and genes encoding plasma membrane cation transporters revealed a genetic interaction between *BMH1* and *NHA1*. These results show that the yeast 14-3-3 proteins and an alkali-metal cation efflux system interact and that this interaction enhances cell survival upon salt stress [Zah12]. To further understand the role of 14-3-3 proteins and the Nha1 antiporter in salt stress resistance, the effect of high external NaCl concentration on the levels of these proteins was studied here. Cultivation in the presence of 0.5 M NaCl resulted in an increased level of both Bmh1-GFP and Bmh2-GFP. This observation is in line with a role of 14-3-3 proteins in tolerance to high environmental NaCl. Moreover, analysis of vacuole sizes revealed larger vacuole size and increased number of vacuolar compartments under increased concentration of environmental NaCl; this result might be a mechanism that the cells employ under stress conditions in BMH1 – GFP cell strain. In order to validate if these results are correct, we propose to perform an experiment in which we stain the vacuoles with a bio-marker. Further analysis using sophisticated feature sets as discussed in Chapter 4 is possible, for example, to recognize the characteristic differences between all the cells cultured in increased concentration of environmental NaCl and those in low concentration of environmental NaCl. Such characteristic differences cannot be seen by standard descriptive analysis. However, such analysis was not relevant in the study performed in the current chapter.