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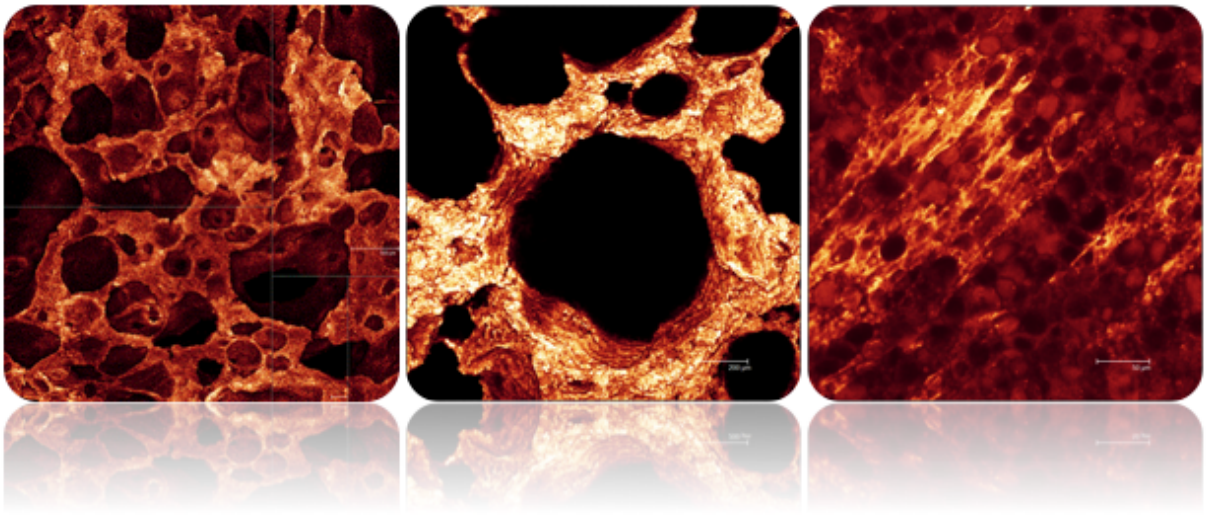

Detection method for salt content and spatial distribution in bread using CLSM

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Title page

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Abstract

A major health concern today is the increasing intakes of salt from many of the food products consumed every day. The high salt content contributes to health issues like raised blood pressure and increased risk for cardiovascular disease. A national project to reduce the amount of salt in several food products in the purpose to increase the health in the population started in the spring of 2017. One of the food products that is included in the project is bread products where different types of spatial salt distribution will be evaluated. The purpose is to reduce the amount of salt without effecting the taste and functionality of the bread.

To verify the tested distribution methods a method has been developed for detection of salt distribution. The method has been developed with confocal microscopy and the sodium sensitive probe CoroNa Green. The confocal microscope is well used in food research and is a good tool to study bread due to the ability to created 3D-projections. Model systems with different complexity have been studied to find limitations and optimized conditions for the probe. The studied systems have been: salt solutions, gelatin gels, dough and bread. The influence of different settings on the microscope and the function of the probe have been evaluated.

The result shows that the probe works well in all systems and that the method is most likely to be able to determine changes in the salt concentration within a sample. 3D-projections have successfully been used to study bread where the structure is clearly shown and it has potential to also visualize inhomogeneous salt distribution. The main parameters to keep in mind are that the probe is sensitive for heating, the diffusion and affinity to the sample and between different components. It is also important to use the same application method, concentration and incubation time for all samples that shall be compared to each other. These parameters influence the responding fluorescent signal and might provide a false result if they are varied between different replicates.

Key words: confocal microscophy, salt, CoroNa Green, bread, dough

Sammanfattning

En bidragande orsak till dagens hälsoproblem är det ökade intaget av salt från dagligen konsumerade livsmedel. Det höga saltinnehållet påverkar hälsan genom att bland annat bidra till förhöjt blodtryck och ökad risk för hjärt-kärl sjukdomar. Under våren 2017 startades ett nationellt projekt för att minska mängden salt i ett flertal livsmedels-kategorier med syftet att öka folkhälsan. En utav kategorierna som behandlas i projektet är bröd där olika typer av spatial, inhomogen, saltfördelning kommer utvärderas. Syftet är att minska mängden salt i produkterna utan att det påverkar smakupplevelsen eller brödets funktionalitet.

För att utvärdera om de testade fördelningsmetoderna fungerar har en metod utvecklats som kan detektera inhomogen saltfördelning. Metoden är utvecklad för ett konfocal-mikroskop tillsammans med den natrium känsliga proben CoroNa Green. Konfokalmikroskopi är väl använt inom livsmedelsforskning och är ett bra verktyg för att studera strukturen i bröd genom att skapa 3D-projektioner. För att hitta begränsningar och optimala förhållanden för proben har modellsystem av olika komplexitet använts. Saltlösningar, geler, degar och bröd har studerats och effekten av olika inställningar på mikroskopet samt probens funktionalitet har utvärderats.

Resultaten visar att proben fungerar bra i alla systemen och att metoden med stor sannolikhet kan detektera skillnader i saltkoncentration i ett prov. 3D-projektioner har med goda resultat används för att studera strukturen i bröd och metoden har god potential att även kunna visualisera inhomogen saltfördelning. De viktigaste parametrarna att ta hänsyn till är att proben är känslig för uppvärmning samt diffusion och affinitet mellan olika komponenter i provet. Det är också viktigt att samma applikationsmetod, probkoncentration och inkubationstid används för de prov som skall jämföras med varandra. Dessa parametrar påverkar den fluorescerande responssignalen och kan ge ett missvisande resultat om de varieras mellan olika replikat.

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1 Introduction

Non-communicable diseases, NCDs, is a major health concern that are categorized into cardiovascular diseases, chronic respiratory diseases, diabetes and cancer. The main cause for the diseases are tobacco use, unhealthy diet, physical inactivity, and high alcohol consumption. About 14 million people every year dies form these diseases and among them does the majority die from high blood pressure, that is one of the main causes for cardiovascular disease [1, 2].

Elevated blood pressure is often caused by a diet with too much salt (sodium chloride), alcohol use, inactivity and obesity [3]. To increase the health of the population by change of diet, one way is to reduce the amount of salt in the food [2]. WHO has a goal to reduce the intake of salt in the main population with at least 30% until 2025 and for Sweden that means a reduction from 10-12 g/day to approximately 7-8 g/day [4]. The reduction of salt will reduce life threatening diseases like stroke and also reduce the number of premature deaths. Reducing the daily salt intake will give effects that are comparable to the benefits from reduced tobacco use and obesity [5].

Salt is an important ingredient and is used in food products to affect taste, processability, texture and safety [5, 6]. The difficulties with reducing the amount to a healthy level are that all of the effects have to be taken into consideration. Salt is also used in almost all kinds of food, with different texture, function and origin. The types of processed food that contains most salt are processed meat, cheese and sauces [7] but the salt content is also high in cereal products which contributes to about 25-30% of the total salt intake [8, 9]. Salt is an important ingredient in bread and contributes to e.g. the flavor, the stability of the dough, the fermentation and the required mixing time [6].

It would to some extent be possible to just reduce the amount of salt in bread, without effecting the functionality and the taste of the product [6, 10]. A critical aspect with salt reduction is to maintain the appreciation from the consumers, which means that products with reduced salt content should not affect the flavor in a negative way. The likeability of the product is important for acceptance from the costumers so it is important to preform sensory studies in combination with projects for salt reduction in specific products. An inhomogeneous distribution of salt particles might provide a product with less salt content without affecting the taste due to an effect called pulsing. The effect come from that the concentration is high in some points and low in some. The total sensory experience gives more salt flavors then a homogeneous distribution. The distribution can be made either by using techniques to encapsulate bigger salt particles or by use sheeting of dough with different salt concentrations [11, 12, 13, 14].

By use of sheeting it is possible to reduce the amount of salt with 25-28% without affecting the total saltiness of the bread. Sheeting can be made either by using salted layers containing fine salt particles or by using a combination of fine salt and bigger, encapsulated salt particles [13, 14]. By encapsulating of salt is it possible could to reduce the salt content with up to 50% without affecting the saltiness of the product [11]. Use of big salt particles that are not encapsulated can give a reduction with up to 25% [12]. For all methods the critical part is to find a good size of either the layers or the particles.

Salts like potassium chloride has a bitter off-flavor that affects the product. In bread, used

in combination with other ingredients like pizza dough, the bitter flavor is masked by other components. This enables the use of other salts than sodium chloride that are better for the health due to that other salts are less reactive in the body than sodium chloride [15]. Combination of salt replacement and reduction of total salt content can enable reduction by up to 25% without giving any changes in the taste [15]. Other kinds of salt than sodium chloride has the disadvantage that they give a bitter taste to the product if the concentration gets too high. Even if those types of salts are better for the health and they don't affect the functionality of the bread it's not possible to exchange all sodium chloride with for example magnesium or potassium chloride [6]. In products like pizza the total salt reduction can be divided by the different components. For pizza the dough is not as sensitive to salt reduction as the mozzarella cheese and tomato sauce when the taste of the pizza is evaluated. By doing this combined reductions the total salt content of the pizza can be reduced with 30% without reducing the saltiness [16].

Different projects have been conducted in collaboration with the industry and academy to develop products with reduced salt content that will be accepted by the consumers. Reduction of salt to a healthy level requires new technical applications. Different solutions have been developed and tested on several different product categories, and projects have been conducted both on a national and international basis. In 2015, 75 countries worldwide had a national strategy to reduce the salt content, that partly rises from WHO's goal that salt intake should be reduced by 30% until 2025 [4]. An example of an international collaboration project is the European project PLEASURE. This was a collaboration between seven different countries and involved both the academy and industry which was conducted between 2012-2014. The project worked with products in both bakery, meat, cheese, sauces/canned fruit and ready to eat meals. They also came up with solutions for both technical and practical approaches as well as conducting studies in sensory analysis [17].

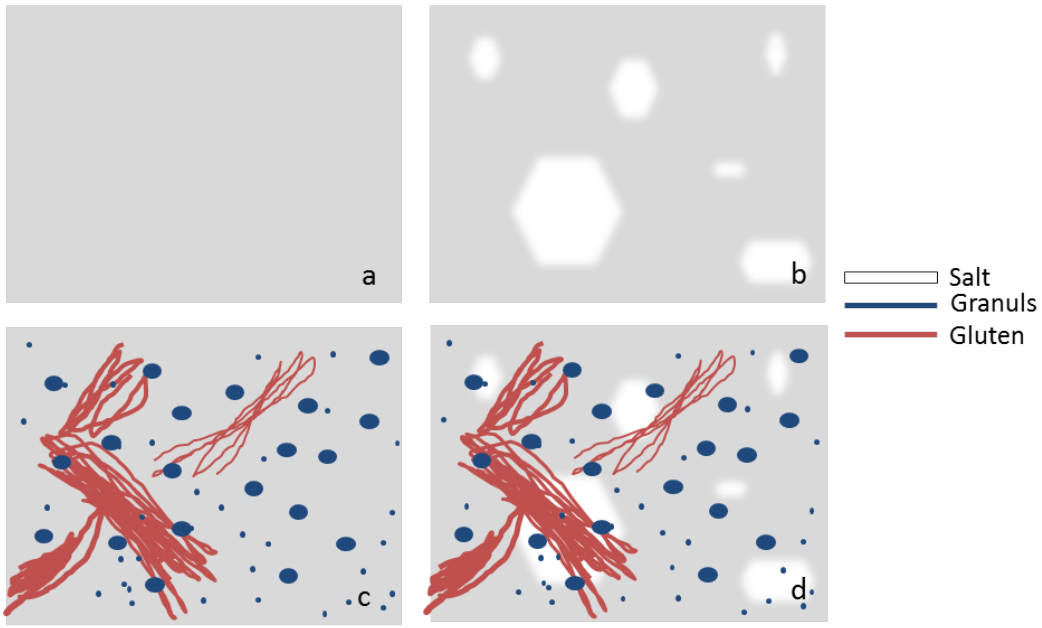
In Sweden a collaboration project between the academy and the food industry started in the spring of 2017 [18]. The aim is to reduce the salt content in four different food categories: bread/cereal products, soup/sauces, processed meat and dry products. The project spans over three years and it is coordinated by RISE. The project is divided into six different working packages and will focus on four different methods for salt reduction within these categories. These methods are: (1) Inhomogeneous salt distribution, (2) Processing solutions, (3) Double emulsions and (4) Flavor combinations [18]. This master thesis is a part of the project in the bread/cereal category as a part of the method to find a specific salt distribution in the food product. The result will be used to validate if different methods for inhomogeneous salt distribution in bread products is working in the way they are designed to do.

1.1 Aim and problem description

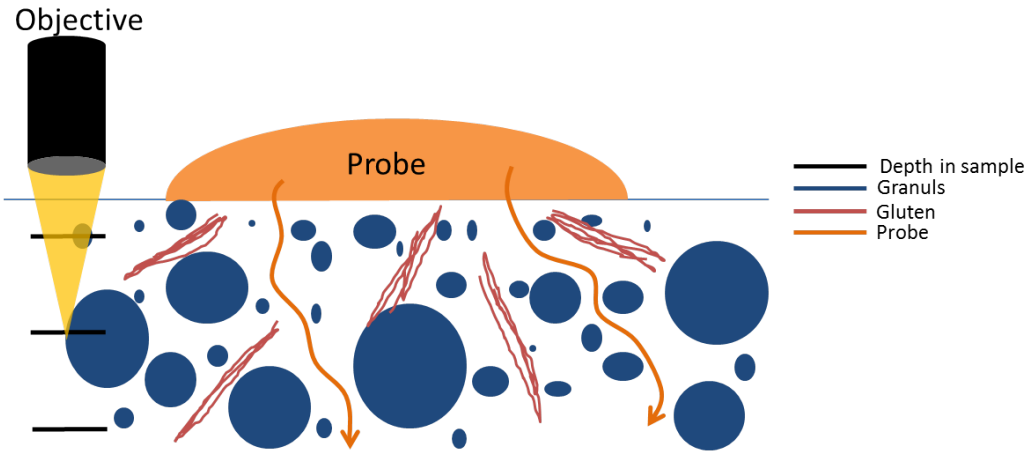
The aim for this master thesis is to develop an image based method to measure the content of salt and to visualize inhomogeneous salt distribution in food systems. The method is based on the use of confocal microscopy in combination with the sodium sensitive, green fluorescent indicator CoroNa Green. The limitations and working conditions for the probe are investigated in both homogeneous (solutions and gels) and heterogeneous (dough) model systems and in bread. The first goal is to evaluate the relative distribution of salt within a sample. The second goal is to be able to determine the exact concentration.

To find a method that works for a complex product like bread the development has to be made in steps. The project has been divided into parts with different complexity of the studied sample. The probe was studied in solutions and gels to evaluate the basic behavior and how it was affected by different settings and external factors in homogeneous systems. An illustration of a homogeneous system with different salt distribution can be found in fig 1 a) a-b. Dough and bread was studied as heterogeneous systems to find changes between a compact and a porous system. The four systems have both different complexity, viscosity and water content that can influence the behavior and mobility of the probe in the sample. The studied probe interacts with free sodium ions and the composition and content of a sample may influence the number of free ions. An example of different components that may influence the response in a dough system is illustrated in fig 1 a) c-d. The complexity of the system may influence both the probe and the salt distribution by hiding the salt and influence by different affinity.

The studied settings were mainly gain and laser power (AOTF) but scan speed, zoom, and line average has also been evaluated. External effects were also studied such as time dependence, depth in the sample and concentration of both the probe and salt. Different application methods were used to study the affinity of salt to different components in the dough and diffusion of the probe into the sample. All of these factors may influence the result and has to be evaluated. Fig 1 b) illustrates the problem with diffusion of the probe and effects of different depth in a dough system. The probe is influenced by the different components in the dough that may affect the time it takes to reach a certain point in the sample. The affinity to other components than salt may also affect how the probe is distributed and how fast it diffuses. The laser light is also affected by the components in the sample. For a sample with compact structure does components above the focus point reflect the light and make the signal weaker. This influences the total depth in the sample that can be studied.



((a)) a and b: homogeneous and inhomogeneous salt distribution in a homogeneous system. c and d: homogeneous and inhomogeneous salt distribution in a heterogeneous system.



((b)) Diffusion of the probe in a dough model system. The different components in the dough influences the diffusion rate. The left side shows the laser light from the objective down to the focus point in the sample.

Figure 1: Visualization of the research problem. a) shows the differences between a homogeneous and heterogeneous system and salt distribution. The salt distribution in a heterogeneous dough system is partly hidden by the overall structure from starch and gluten. b) shows the probe diffusion and how the laser light is affected in a complex sample.

2 Theory

This chapter will consider the technical effects salt has on bread and dough. The used microscope and important settings will be described along with the sodium sensitive probe CoroNa Green. Other techniques and probes will be discussed as well.

2.1 Effect of salt and inhomogeneous salt distribution in dough and bread

The four main ingredients in bread dough are flour, water, yeast and salt [9]. Addition of salt affects dough in different ways including processability, the structure and behavior as well as the components within the dough. The effect that salt has on bread and dough is not necessarily good or wanted and some of the positive effects can be achieved by other components [8, 9, 19, 20, 21, 22]. Salt bring out other flavors in the bread and increases the sweetness as well as masks off-flavors and metallic taste. Due to that it is not possible to remove all salt without decreasing the flavor [9].

When wheat and water are mixed, a viscoelastic dough is created due to the two types of proteins in the gluten – glutenin and gliadin. Glutenin forms the network and gliadin affects the viscosity by interaction with glutenin via non-covalent hydrogen bonding. Salt affects the processability of the dough by contributing to the formation of a fibrous structure. Without salt, non-viscoelastic aggregates are formed by gluten and water. If salt is present during the mixing of the dough when proteins unfold, the formed network will be tighter [19]. The rheology of the dough is not significantly affected by the salt content, but if salt is completely removed both the resistance to extension and extensibility will be reduced. Both the elastic and viscous modulus are increased by increased salt content [8].

Salt affects the hydrophobicity in dough by inducing charges to the gluten polymers [8, 9]. The gluten chains repulse each other if no salt is present due to a net positive charge. This gives a fast hydration of the gluten which results in poor interaction between the gluten chains. The strength of the dough depends on the interaction between the proteins to form a strong network and this doesn't happen if no salt is present. The addition of salt shields the charges on the gluten chains that result in an increased interaction and decreased hydration [9]. Salt delays the gelling and hydration of the starch granules, which may reduce the cross-linking rate in the proteins [8]. The connection is not clearly understood but could be connected to the water activity that is decreased when salt is added. Lower water activity requires higher energy for physical reactions that results in slower cross-linking formation [23]. The molecules in water can be structured in different ways that give different properties and possibilities for interaction. If the molecules are well structured, they have high ordering and the ordering affects the interaction between the gluten chains. If the ordering is high, the ability for the gluten to interact with each other hydrophobic interactions increases. The ordering of water molecules increases if salt is present that gives a tighter structure in the water phase with charged areas towards the salt ions [9]. If there is unbounded water in the dough, there will be an increase in stickiness. That is a problem in the bread industry because sticky dough is hard to handle and disturbs a continuous production. When it comes to salts involvement in the stickiness the results are conflicting [21]. Salt has an influence in the hydration of gluten [8, 9, 21] and the viscosity [19, 21] which are factors that affect the stickiness. This indicate that salt can influence the stickiness of dough [21].

The proofing of dough contributes to an increase in volume because of gas production during yeast fermentation. Salt is an inhibitor for the yeast growth and can be used as a regulator for the proofing of the bread dough. This is due to an increase in the osmotic pressure and the ionic strength [8, 9, 20]. The changed environment affects the cell membrane of the yeast, decreases the growth rate [8] and the viability of the cells [20]. By adjusting the salt content, both the proving rate and the proving time can be adjusted [8, 9, 20]. Even if the production rate of gas is lower in a dough with high salt content, the gas holding capacity is higher [8, 20]. The salts have a strengthening effect on the gluten network that increases the ability to keep the produced gas in the dough. [8] If no salt is added the result will be a dough with high proofing rate that gives a poor structure and sour taste [9]. Salt addition is more harmful to yeast in sweet dough because sugar also effects the osmotic pressure. The combination of salt and sugar in the dough decreases the water activity and the effective concentration of salt increases [20].

The strength and volume in bread is affected by the salt content due to the effect on the gluten network. The same effect can be achieved by using a flour with higher protein content and reduced amount of salt [10]. The volume is partly affected by the size of the gas bubbles in the bread, and in a bread with salt the bubble size is smaller which would give a lower volume [22]. At the same time studies show no significant effect on either the volume or the hardness of the bread with or without salt [8]. This conflicting result might be explained by use of different recipes and studied salt levels, but it also indicate that the volume is not only affected by the salt content of the dough. Salt is necessary to get an even crumb structure [8] and it affects the firmness of the crumb due to the effect on gas bubbles. The firmness is correlated to the staling in the way that a firmer bread has increased staling rate [22]. Different kinds of microscopy techniques can be used to study both the structure and the influence of ingredients in bread and dough. For this project confocal laser scanning microscopy, CLSM, is used.

2.2 Confocal laser scanning microscopy, CLSM

CLSM uses fluorescence and enables projections in 3D by scanning the sample in different layers. It was in 1985 adapted to biological samples by Brakenhoff, Wijnaendts and Carlsson. It is now a well-used method to study structures in many types of biological samples including food products. The possibility to scan different layers of the sample makes it possible to study border surfaces within the structure [24]. There are two different mode for the CLSM, reflection and fluorescent mode. The reflection mode studies the topography and surface of the sample and the fluorescent mode studies structures within the sample. This is the most common mode and it is used in this project. The fluorescent mode is induced by a laser beam that excites molecules within the sample and releases fluorescence that are detected [25].

There are different laser diode arrangements that produces laser beams of different wavelengths. They are mainly in the spectra for visible light, but it is also possible to use UV or IR lasers. Lasers that uses semiconductor materials usually has a wavelength of 300-460 nm, argon lasers has wavelengths from 450-565 nm and helium lasers has wavelengths from 543-643 nm. The most common is to use lasers in the range 488-647 nm [26]. The laser light is monochrome, which means that there is only one wavelength used to excite the molecules in the sample [25]. The excited fluorescent molecules in the sample release fluorescence emission

that is detected. A laser has one specific wavelength and it is possible to use several lasers simultaneously to study different components in the same sample that are excited by different wavelengths. If different lasers are used it is necessary to make sure that the emission spectra don't overlap too much and create a false response signal, called cross-talk [26].

It is necessary that fluorescent molecules are present for the microscope to work. The only detectable signal is the fluorescence and this results in low noise from other components in the sample or by physical disturbance [27]. The fluorescence can be achieved either by auto-fluoresce or by use of a fluorescent dye. The auto-fluorescence in cereal products comes mainly from polyphenols like lignin or by ferulic acid in the cell wall. The fluorescent dyes contains molecules with a fluorocrom that gives a signal when the molecule binds to the studied component in the sample. There are two types of dyes that either has a covalent or non-covalent bonding to the fluorocrom. The covalent bonding, also called labeling, is mainly used in multi-phase systems. The fluorocrom is bound to a marker molecule like an antibody with a covalent bond. This molecule then binds to the studied compound in the sample, like to a specific site at a protein. These types of dye are good to use in multi-phase systems since they are compound specific. If the fluorocron don't have a molecule bound to it the dye is called non-covalent or staining. In this case the fluorocrom spread in the sample depending on affinity. The spreading depends on the structure of the fluorocrom and its solubility in e.g. water and it is not targeting a specific compound. These dyes are good to use in single-phase systems or for study proteins in multi-phase systems [25].

To scan the structure a laser beam is moved stepwise over the sample in the x/y-direction, fig 2. Before hitting the sample, the laser goes through a beam splitter (fig 2.5) and the objective (fig 2.2). The emission is collected by the objective and focused in a pinhole (fig 2.5) that removes all out of focus light. After the pinhole, the signal is translated in the detector (fig 2.6) to a digital signal that is read by the computer software. Due to that the out of focus signals are reduced by the pinhole it is possible to study relatively thick samples and also dynamics within the sample [26, 27].

The pinhole makes it possible to provide a detailed image of a thin layer in the sample without disturbance from out of focus parts. The intensity in each point of the image is determined by the amount of light that is transmitted by the pinhole for the same position in the sample [28]. The size of the pinhole is critical to reduce all out of focus signals and to make sure that enough light passes on to the detector. If the size of the pinhole increases, the signal comes from a wider area and depth that increases the out of focus signals and results in a blurry image [29]. This is one advantage that the confocal has compared to a light microscope where the noise signals aren't reduced. The advantage of studying just one small piece at a time is that the light is focused in a specific spot which gives less stray light and both the focus and contrast gets improved [25]. The scanning is also made one row at a time in the x/y-direction compared to the light microscope that studies the whole frame simultaneously [25].

The resolution is used to compare microscopes and it depends on both the wavelength and the numerical amplitude (NA) of the objective [25]. The numerical amplitude is an objective specific constant that affect the amount of emitted fluorescence that can be collected. It also depends on the refractive index of the medium between the objective and the sample [28]. Compared to the light microscope the resolution can be increased with a factor 1.4 for the confocal [25]. The size of the pinhole does also influences the resolution in the confocal microscope and a small pinhole gives the best resolution and the best optical sectioning

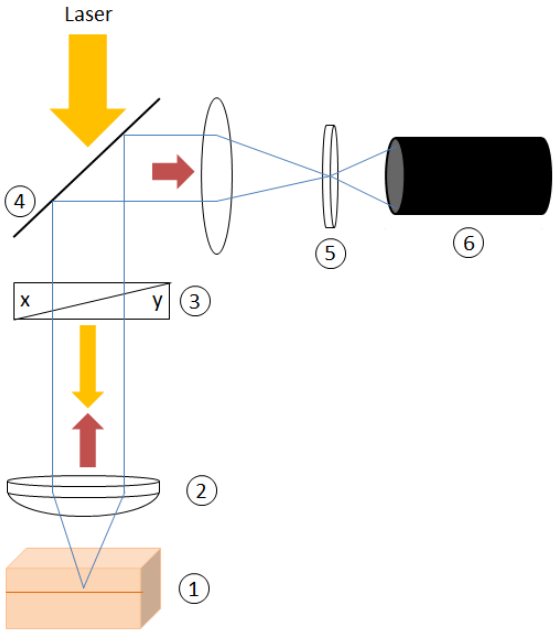


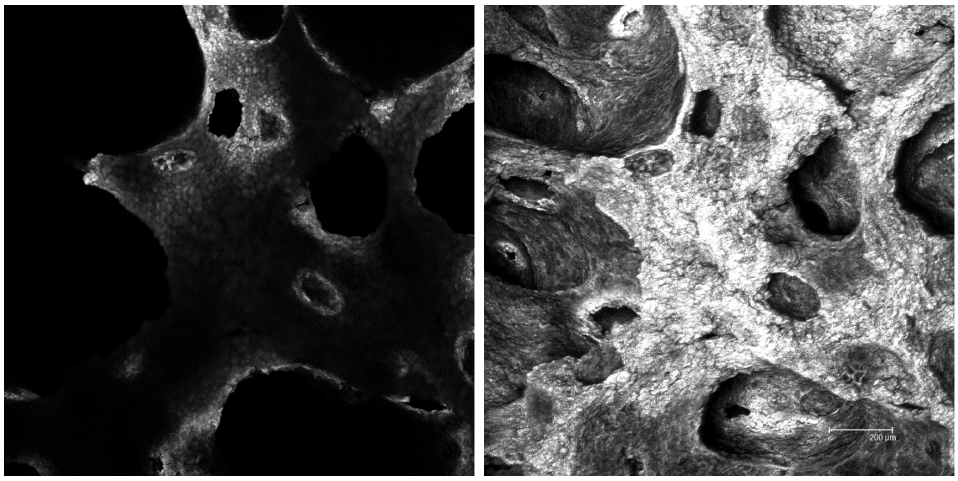
Figure 2: Redrawing from [26] showing the important parts of the CLSM. 1) Focus plane in sample, 2) Objective, 3) Scanner, 4) Beam splitter, 5) Pinhole, 6) Detector

[25, 28]. If there is very limited light in the sample it is possible to increase the pinhole to get more light into the sample, but the consequences are that the out of focus signals will increase [28].

The confocal has both lateral and axial resolution [25] that provides high focus and fine adjustment of small steps in the z-direction to create a series of images. The series can be used to create a 3D structure that contains information from different focus planes [25, 27]. By moving the focus plane in the z-direction in define steps it is possible to create a series of optical sections in 2D that are put together to a 3D projection [27]. Displaying multi-dimensional images provides better understanding of the studied structure. If the projection is created or edited the wrong way may structures in the sample be either amplified or attenuated compared to the reality. The preferred outcome should be a clearer and realistic picture of the structure in the sample compared to the simpler 2D image, see fig 3 [30].

The movement of the focus plane in the z-direction reduces the blurry imaging that otherwise occurs if images of high depth is taken. In the confocal every plane in the structure are scaned separately with good focus and are put together to a 3D structure in the computer software [27]. To get the optimal distance between each image the z-value of the objective is used, this value determines the depth that the objective covers in one picture. The value is calculated by the equation 1. The projections can be used to create reconstructions of the structure based on either structure or volume [27]. The 3D-projection can be viewed as a deep image, 3D-image or animation with rotation. It is possible to study a depth of about 20-50 μm in dough [25]. The images in 2D vs 3D gives very different information about the sample and are used for different purposes. A comparison between a bread structure in 2D versus 3D are illustrated in fig 3 where the structure is clearly shown in the 3D image and for the 2D image is it hard to understand what the image shows.

$$z = 1{,}4 * \frac{\lambda}{NA^2} \tag{1}$$



((a)) 2D image of bread **((b))** 3D-projection of bread

Figure 3: Images of one single layer in the 3D-stack and the full 3D-projection of a bread sample.

Swelling of starch granules in a dough during heating can be studied by use of a heating stage. The increase in temperature can be set and images can be taken at specific temperatures. In this way it is possible to follow what happens in the dough during the heating process [31]. Phase separation, aggregation and both effects of heating, cooling and mixing can be studied with use of stages and image-taking over time [27].

The microscope has many applications and provides a noise free image, but it also has some limitations. The microscope works with fluorescence and can only detect a signal if there is some kind of excitation of molecules in the sample upon contact with the laser beam. For most samples some kind of fluorescent stain is required [26, 27]. The staining process may affect the sample in ways like swelling/solubility because most of the stains are liquids. Several stains are also sensitive to the laser beam and can be bleached if the same spot in the sample is scanned to many times [27]. Another problem with the stains is that they in many cases are non-specific for the studied component. This means that a fluorescent signal may be achieved from other compounds in the sample that provides a false result [32].

The microscope has some other limitations beside the staining. High resolution can be achieved by objectives with high numerical amplitude. This requires short working distance, which limits the depth that can be studied. The transparency of the sample affects the penetration of the laser. In a transparent sample it is possible to study a deeper area since the laser beam is not disrupted and reflected [27]. The surface of the sample has to be flat to get an even signal. This can be hard to achieve when either sticky samples like dough or when porous samples like bread are studied [26]. The laser beam goes through different medias with different refractive indexes on the way from the objective to the focus point. The largest change in refractive index is between the cover glass and the sample. In this interface will the laser beam be refracted that affects the location of the focus plane compared to an ideal system. The refraction does also result in some reflection of the light and can change the

wave front and spread the focus. The refraction is most likely to be a problem in objectives with high NA [33].

2.2.1 Settings of parameters in the CLSM and how it effect the image

There are several settings that influences the brightness/clearness of the image. Some settings are of greater importance than other and several contribute to get a similar output. For the leica confocal TCS SP2 and optimization for salt detection is excitation wavelength of 488 nm suitable. The most important parameters are: format of the image, laser power (AOTF), zoom, scan speed, gain/offset and line/frame average. These parameters have been evaluated and the levels has been optimized.

The laser level can be changed in two different ways: by changing the actual power that comes from the laser and by changing the acousto-optical tunable filter, **AOTF** [34]. The AOTF is an optical crystal where light enters at one specific angle and exits at a specific diffraction angle. This makes it possible to choose one single wavelength of the diffracted light. Usually the AOTF are configured to use the light in the laser beam. The response from the AOTF comes within microseconds that make it suitable for adjusting the laser power [35]. The amount of light that passes the crystal can be controlled by changing the amplitude (the specific position) of the crystal. The amplitude of the crystal is graded in % from 0-100% depending how much of the light from the laser that is passing through the crystal [34]. The laser power has only been changed by the AOTF.

The image **format** decides how many pixels that will be measured in each row, and how many rows that will be scanned. The image size is fixed so the format determines how big each pixel are. It also influences the pixel dwell time, how much light that goes into every pixel. With a high dwell time more photons will be released in every pixel and a stronger signal will be achieved. The format is also influencing the resolution and image raster. A raster point is the area of each pixel and it is defined as the size of the total scanned area divided by the number of points in each scanned row. If the distance between the raster points are to low, it is necessary to zoom to avoid losses. The resolution is determined by the wavelength of the laser and the numerical amplitude of the objective, eq 2. [34]

$$resolution = 0{,}4 * \frac{\lambda}{NA} \tag{2}$$

The **zoom** gives more details to the image and contributes to the magnification. An image with more zoomed still uses the same number of measuring points to scan each row of the image. This means that if the zoom is increased from 1 to 2 the same number of pixels is used to scan half of the length. This give a 4 times better magnification and a better resolution because the same frequency is used to scan a smaller area [34, 36]. The frequency, (**scan speed**) is connected to the dwell time together with the format. A longer dwell time gives a better signal-to-noise ratio, but this also increases the risk for bleaching. To avoid bleaching from to low speed the signal-to-noise ration can be improved by using different types of **average**. The whole frame or each row can be scanned from 1 up to 8 times and the average intensity of each point is displayed in the final image [34].

The final way to influence the brightness of the image is to affect the detector. This can be made by adjusting the parameters **gain** and **offset**. The gain modifies the amplification of

the detected signal, which influences the brightness, and contrast of the image. The offset affects the threshold, which is the darkness of the image and means that only signals with higher value than the threshold will be detected [34].

2.2.2 Microstructural analysis of bread and dough using CLSM

Confocal microscopy has been used for study several different aspects of dough and bread. Both structures of specific component as well as the effect of different treatments and preparation techniques have been evaluated.

Gluten molecules and the gluten network can be studied during different stages of dough development and the mixing. With staining of the gluten, it is easy to follow the changes in the structure with the microscope. Both the effect of different water and salt content on the gluten network and dough development in a sour dough can be studied [25]. By staining different components in the dough, like the gluten and starch separately the interactions and effects on each component can be studied simultaneously. This is useful if the dough is studied during proofing where development of the dough can be studied over time and changes in each component can be investigated [37].

Simulation of bubble formation during proofing in dough and during baking can be complemented and evaluated by comparing to experimental data. The size and amount of bubbles can be studied in the microscopic images and be compared to the calculated values in the simulation [38]. The effect of storage compared to fresh bread [27], dough structure development as an effect of freezing and mixing can also be studied [25]. Different proofing time and effect on different ingredients in frozen dough [39] can be studied as well and the structure before/after a process or by use of different techniques. Differences in the bread crumb and crust for evaluation of crispness can be studied by comparing the size of the gluten network and starch granules. This can be used to find out how gluten contributes to the crispiness of the crust [40]. The effect of different fat content can be studied by visualizing differences in the gas bubble formation and void spaces in bread dough with different amount of fat [41].

The microstructure in bread with inhomogeneous salt distribution by coated salt granule can be visualized with the confocal microscope. The confocal imaging can be used to verify if the distribution method works as planned and to evaluate changes in the structure with different sizes of the coated granules [11]. The effect of different salt content on the gluten network can be visualized by staining of the gluten. The structure can be compared to results of different rheological measurements to find connections between the effects on the dough and the structure. The results can be used to find out what influence the salt has on the structure that contributes to changes in the rheology like stickiness and strength [21].

2.3 Fluorescent probes for detection of sodium ions

To make the sodium visible with the confocal microscope a fluorescent indicator (probe) has to be used [32]. The probe binds to the sodium ion and is excited by the laser beam and creates a fluorescent signal that is detected. The probe has an ion-binding site for the sodium ion and a fluorocrome that induces the fluorescent signal during excitation [32]. A good sodium probe is supposed to be selective towards sodium and have a low affinity towards other similar

ions [32, 42]. Both the fluorescent properties, specificity, chemical properties and affinity has to be taken into consideration when choosing a probe [32].

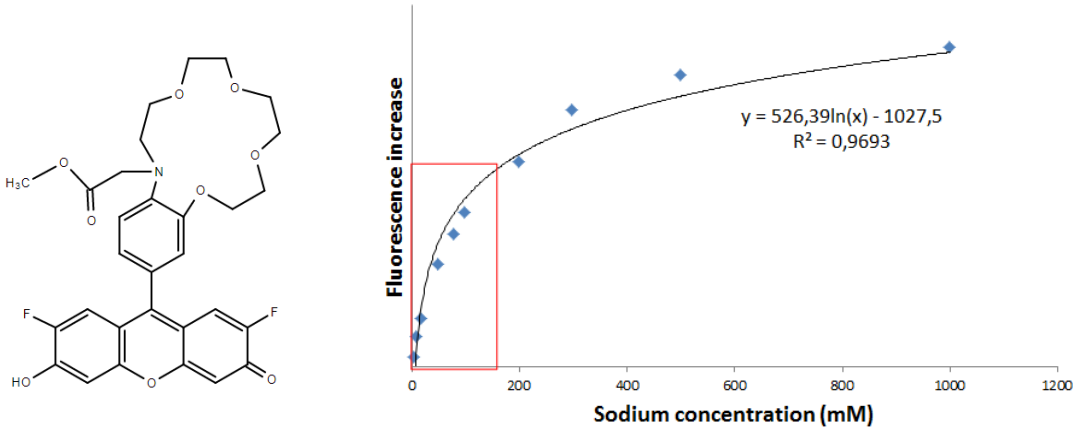
A large increase in the fluorescent response upon binding to sodium ions as well as a large increase in the emission intensity is required to get a good and reliable result [42]. The affinity for the sodium to the probe is determined by the dissociation constant k_d and is specific for every probe. The constant may differ depending on the environment, especially for *in situ* or *in vitro* studies. The constant determines the concentration range of sodium that can be detected, and the detection range is about 0.1-10 times the k_d value [32]. It can be calculated from eq 3 for an area with linear increase in fluorescence [43]. In the equation is F the fluorescence and min/max indicate the fluorescence at the lowest and highest detectable ion concentration [43]. The microscope that has been used in this project has lasers that can work in the excitation range from 458-633 nm. The probe needs to have the excitation maximum within this range and because of that can't any probes for UV spectra be used. The probe also has to be able to work with the confocal microscope and be water soluble. The chosen probe CoroNa Green has been compared to the other different existing sodium probes Sodium Green, SBFI and ANG1.

$$K_d = \frac{[Na^+] * (F_{max} - F)}{(F - F_{min})} \tag{3}$$

2.3.1 CoroNa Green, a suitable probe for detection of sodium ions in bread

CoroNa Green was developed as a complement to the other previous existing sodium specific fluorescent dyes SBFI and Sodium Green. It consists of a bensoaldehyde with a crown ether, specifically a 15-crown-5 compound with 15 atoms in total and 5 electronegative atoms. The bensoaldehyde is the fluorophor (bottom part of the molecule), see fig 4 a). The structure of the molecule shows better characteristics then compounds containing both larger and smaller crowns [44]. The total size of the molecule is smaller compared to other similar molecules [32] with a molecule weight of 586 [43]. How the sodium ion binds in to the molecule is not stated but there are a net negative region in the middle of the crown structure due to the oxygen and nitrogen atoms that might attract the sodium ion. The probe gives an increase in fluorescent response with increased sodium concentration. The increase in signal is not linear to the salt concentration [44], as shown in fig 4 b) and it is important to find the concentration interval for the specific condition that gives linear response to be able to interpret the results [42]. But *in vitro* studies show an almost linear response in emission fluorescent for concentrations up to 170 mM, which corresponds to 1% salt in a water solution [45].

The selectivity for sodium over potassium is important to know especially when cells are studied due to the similarities of the ions and the presence of potassium in cells. This is used as a reference to compare different probes by evaluation of the risk to give a response for the wrong ion. The selectivity in CoroNa for sodium is 4 times higher than for potassium, which is in line with other molecules with similar structures [44]. The selectivity is on the other hand lower in CoroNa compared to both SBFI, Sodium Green and ANG-1 and 2 [32]. The dissociation constant k_d is another way to evaluate the probe and for CoroNa Green the constant is between 80-100 depending on the environment [32, 44]. The excitation and emittance wavelengths are 492 and 516 respectively which means that the 488 nm argon laser can be used with good results [43]. The probe is fluorescent within the visible range and has



((a)) Structure of the CoroNa Green molecule

((b)) Intensity change with increased sodium conc in MOPS at λ_{em} aprox. 515 nm

Figure 4: Structure and data are modified from [44]

to be protected from light during storage and it should also be stored cold [43].

CoroNa has been used in several studies where salt concentration and distribution has been studied. Most of the research has been in different kind of cell with both human cells, animal cells and plant cells as well as intra-cellular and extra-cellular studies. The different studies has different conditions and used different concentration of the probe spanning from 2-100 μM and also different salt concentrations has been used in the studies [42, 46, 47, 48]. The probe has been used for salt distribution in bread with the concentration 30 μM and the salt concentration differed from 1-2% on flour base [11].

2.3.2 Other probes that can be used for detection of sodium ions

A comparison between the four sodium probes CoroNa Green, SBFI, Sodium Green and ANG-2 can be seen in tab 1. The molecular weight, dissociation constant and selectivity for sodium over potassium is shown. The table shows that CoroNa Green has lower molecular weight compared to the other three because it only has one fluorophor. The selectivity for sodium over potassium is lower for CoroNa Green compared to the others due to different molecular structures especially in the crown structure [32]. The dissociation constant is higher for CoroNa Green which indicates that higher concentration changes can be detected, but another probe should be chosen if small changes are studied [42]. The excitation and emittance appear in a wide range of wavelengths but with a maximum at a specific wavelength. The excitation wavelength determines the laser that can be used to excite the probe. The distance between the maximum excitation and emittance wavelength should be big enough to just give a small overlap between the two response curves [26]. The excitation wavelengths show that both CoroNa Green and Sodium Green can use the 488 nm laser. That laser could also work for ANG-2, but the most optimal laser for that probe is 514 nm, for SBFI does a UV laser has to be used [32].

SBFI, sodium-binding benzofuran isophthalate, is the first and most common sodium probe

Table 1: Comparison of fluorescent sodium probes

parameter	CoroNa Green	SBFI	Sodium Green	ANG-2
Molecule weight	586	907	1668	1084
k_d (mM)	80-100	18-26	6-21	34
Selectivity sodium	4	18	41	20
λ_{ex} (nm)	492	340/380	507	517
λ_{em} (nm)	516	505	532	540
References	[43, 44]	[32, 45]	[32, 49]	[42, 50]

[32]. The structure of the molecule is similar to the common calcium indicator Fura-2 and has been used for studies in various types of cells [45] and also for studies of inhomogeneous salt distribution in bread [12]. It has two fluorophores which makes it rather big and bulky compared to CoroNa Green [44] and has a molecular weight that is almost the double, see tab 1 [45]. Compared to CoroNa Green is SBFI better at detecting small transients in the sodium concentration at low concentrations. But CoroNa Green is better at determining larger transients at higher concentrations of sodium [45]. It also has higher selectivity for sodium, see tab 1 but the disadvantage is that the probe is fluorescent in the UV-range and not by visible light [32, 45].

Sodium Green is a rather big molecule with a similar structure as several probes for both calcium and magnesium detection [51]. Similar to SBFI does Sodium Green has two fluorophores which makes it rather big and bulky compared to CoroNa Green [43, 49, 51]. It has rather low increase in fluorescence upon binding to sodium compared to the received signal when no sodium is present [44]. The excitation is within the visible range with maximum at 507 nm and the emission maximum is at 532 nm [49] which makes it suitable to use with the 488 nm argon laser [32]. Compared to the other probes, Sodium Green has higher selectivity for sodium than for potassium. The k_d approximately in the same range as for both SBFI and ANG-2, see tab 1 [32, 49]. The change in intensity between Sodium Green bounded to sodium ions compared to unbounded ions are 6.7 times higher for the bounded one [51]. Sodium Green is well suited for fluorescence lifetime imaging microscopy [51] but has also been used for studies with confocal microscopy in different cell studies [52, 53]. No studies has been found where Sodium Green has been used for bread or food studies.

ANG-1 and 2, asante NaTrium Green-1 and 2, are newer then the other probes and similar to CoroNa Green they have one fluorophor but they are still in the same size as SBFI and Sodium Green, see tab 1 [32, 42]. They differ a bit in the structure and the ANG-2 has better fluorescent properties then ANG-1 but both of the probes has the same excitation and emission wavelengths and have similar k_d value [32, 42]. They are both rather new probes and has only been used in a few studies [42]. In cell studies, ANG-2 has shown better reliability for detection of small changes in the sodium concentration compared to CoroNa Green [42]. It has been used successfully with fluorescence lifetime imaging microscopy both *in vitro* and *in situ* [50] but no studies has been found where it has been used with either confocal microscope

or with food products.

2.4 Additional microstructure techniques suitable for analysis of bread and dough structures

Scanning electron microscopy, SEM, uses an electron beam and study the sample in vacuum to prevent interaction with the electron beam and gas molecules. An electron probe is used with a current of about 10^{-12} - 10^{-10}A that provides a good signal to noise ratio. The electron probe is scanned line by line across the sample and all the signals that is generated after that the electron beam has hit the sample is recorded. The electron beam hits the sample from above and after hitting the sample electrons are spread in different directions on both sides of the sample. The microscope can detect two different types of response signals, either backscattered electrons or secondary electrons. Backscattering is the reflection of electrons from the electron beam and secondary electrons are excited electrons from the probe at the sample surface when hit by the electron beam. The backscattered electrons have higher energy then the secondary electrons. One of the advantages of SEM is the depth of focus, which is about 100x greater than that of an optical microscope. It has good contrast, straight forward sample prep and compared to X-ray micro analysis it has good capacity for quantitative analysis in a specific location at the sample surface. The sample can be studied in the scale from mm down to 100 nm. The microscope is limited by the requirement of vacuum and that it only studies the sample surface [54].

SEM has been used to study the effect of storage on the microstructure in sourdough bread. Before analyzing the samples, they have to be dried and coated to create a surface that can be detected by the electron beam. Images of the dough before baking with different baking techniques was compared to images of the same techniques after different days post baking [55].

Environmental scanning electron microscopy, ESEM, is a variation of SEM that studies the sample in the presence of a gas. That makes it possible to study liquids and humidified samples without harsh preparation. The method makes it possible to study samples without use of e.g. conductive coating and freeze-drying. The most common gas to use is water vapor, but other gases can be used if the sample is sensitive to or may interact with water. The used gas in the sample chamber gives scattering of some primary electrons that impacts the surrounding of the studied surface. X-ray microanalysis in ESEM is a way to qualitatively study the elements that are present [56].

Light microscopy uses transmitted visible light to study a sample and requires thin samples. The sample has to be thin enough to enable transmission of enough light to capture the structure. For brittle materials like bread fixation and embedding might be needed to prepare a section of a sample of about 1μm. The fixation and embedding can be performed in different ways depending on the sample and the studied compound. The microscope can with good result be used without staining the sample which is an advantage compared to other microscopic techniques. For bread can the amylose and amylopectin in the starch granule be studied as well as the relation of starch compared to for example fat and proteins. Dough is more compact than bread and requires thinner samples to enable transmission of the light [57]. The samples may be prepared with embedding like for bread or by cryostat. In a

cryostat are thin slices cut during freezing and thinner samples then with embedding may be prepared. For dough is the light microscope good to study amylose and amylopectin in the starch granules [57, 58].

X-ray tomography uses an X-ray beam that is focused on the sample and a shadow image reflecting X-ray attenuation along the beam path is recorded. The sample is rotating and analyzed by tomography. It has high penetration power and is not limited by complexities in the surface and is also non-invasive to the sample and can be used without any sample preparations. When the X-ray beam interacts with the sample it can result in transmission, absorption or scattering. The x-ray tomography is imaging the transmitted photons through the sample. It produces a 3D projection by taking images from different directions of the sample and put them together to a single projection. The technique can be used to study both dough structure, baking and bread structure [59]. The method has been used to study salt granules in bread dough to implement inhomogeneous salt distribution. The X-ray studied different coatings effect for dissolving of the salt particles over time in the bread dough [14].

3 Experimental methods

The working methods for the studied systems is described in this chapter. The method for homogeneous systems (solutions and gels) and heterogeneous systems (dough and bread) are described separately. The complexity differs in the model systems as well as the focus and method between them. The focus in **salt solutions** was on the effect of microscopic settings, concentrations and environmental parameters. For **gelatin gels** was changes between a solid and semi-solid system the main focus. In **dough** were physical and environmental parameters in a complex heterogeneous system evaluated. A method for 3D-projections was created by studying **bread**. For all systems, the limitations for the probe was studied and a suitable working method for each system was optimized for Leica confocal TCS SP2.

3.1 Preparation of the *CoroNa Green* probe solutions

A powder of CoroNa Green was dissolved in dimethylsulfoxide (DMSO) to a concentration of 2mM stock solution. The stock solution has been further diluted with DMSO or ethanol to the preferred working concentrations. The solutions deluded with DMSO has been diluted to concentrations from 40-200 μM and was used for the homogeneous systems and partly for the dough. It was diluted from the stock solution according to tab 2 and the volumes was calculated by eq 4.

$$C_1 * V_1 = C_2 * V_2 \tag{4}$$

The solutions with ethanol was diluted to a concentration of 30 μM according to eq 4. It has been used mainly for the heterogeneous systems due to that ethanol evaporates and can be dropped on the surface of the sample without destroying the structure. All probe solutions has been stored in the freezer and kept in foil while use to protect from light.

Table 2: Added DMSO and probe stock solution to get the used probe concentrations

Final conc (μM)	$V_{DMSO}(\mu l)$	$V_{stock}(\mu l)$
40	980	20
100	950	50
120	940	60
140	930	70
200	900	100

3.2 Preparation and evaluated aspects for homogeneous model systems

To investigate how the probe behaves and what limitations that has to be taken into consideration later on was the first part of the project to study homogeneous systems. Salt solutions was studied as a liquid system and gelatin gels was studied as a semi-solid homogeneous product. Gel systems acts as an intermediate step between a liquid and a dough with higher

complexity then a salt solution and simpler structure then a dough. The focus for the homogeneous systems was to study the effect of different settings and the response to an increase in concentration of the probe and salt.

3.2.1 Sample preparation

Salt solutions: Solutions of different salt concentrations was prepared by dissolve 99.9% NaCl with a diameter of 0.1-0.3 mm in distill water. Dilution series were made according to table 3 where series 1 was used to investigate different settings and series 2 was used to study changes in intensity depending on the salt concentration. The salt solution and probe solution were mixed 50/50 which diluted the salt and probe with 50%. All solutions diluted to a concentration double the one in the final test sample and the final concentrations for all salt solutions can be found in table 3. One drop of the salt- and probe-solution was added to a small metal cup as shown in fig 5 a) by a 1 ml plastic pipette and thoroughly mixed before analysis.

Table 3: Salt solution series was created to study how different setting affected the response for different salt concentration in the interesting interval

Series	Created solutions (% salt)	Final conc (% salt)
1	0.2, 1, 2	0.1, 0.5, 1
2	0.1, 0.2, 0.6, 1, 2	0.05, 0.1, 0.3, 0.5, 1

((a)) Prepared sample of a solution in a metal cup

((b)) Prepared sample of gels, both piece of gel with probe drop on top and gel mixed with probe in a metal cup

Figure 5: Samples of solution and gels ready to analyze.

Gels: Gels with different gelatin and salt content was prepared by heating gelatin, salt and water to 70°C. Gels containing final gelatin concentration of 4 and 8% and 0.1 and 0.5% of salt was prepared. The gelatin and salt were added on percentage of the water. The probe was applied in two ways: dropping on top of the gel and mix the warm gel solution with the probe, like the salt solutions. The probe was only dropped on top of the gel to study the diffusion and the preparation details can be found in table 4. The gels were prepared in advanced and cooled down before the probe was added. In the first gel a defined volume was cut out and placed on an object glass in fig 5 b) and in the 2^{nd} and 3^{rd} the gel was prepared in a small metal cup as demonstrated in fig 5 b). For the part when the gelatin

solution was mixed with the probe solution the concentration of gelatin and salt was double the amount in the test samples, due to deletion by the probe solution. This method was used for investigation of different settings and conditions in the gels and the preparation method can be found in table 4. The gel/probe solution was thoroughly mixed in a similar metal cup as used before, see fig 5 b), and 2 drops of each solution was added to the cup by a 1 ml plastic pipette.

Table 4: Tested preparation methods for gels. Two gels with inhomogeneous salt distribution and two with homogeneous.

Parameter	Drop on gel 1	Drop on gel 2	Drop on gel 3	Mix in gel
Gel status	cold	cold	cold	warm
V_{gel}	60mm^2	45ml	0.5ml	2 drops
Added probe	20μl	30μl	30μl	2 drops
C_{probe} initial	100μM	120μM	120μM	120μM
C_{probe} final	NA	NA	13.75μM	60μM
Probe overflow	0.015g	0.019g	-	-
Incubation time	20 min	20 min	60 min	40 min (gelling t)
Probe distribution	inhomogeneous	inhomogeneous	homogeneous	homogeneous

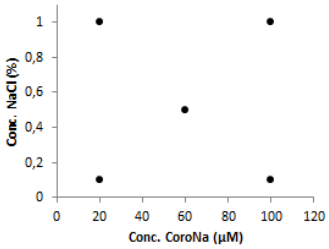
3.2.2 Design and optimization of settings for homogeneous salt content and distribution

Salt solutions: To minimize the number of parameters to optimize it was decided to use fix values for: format (512*512), zoom (4 times), line average (8), objective (20x water objective) and offset (-0.1). The parameters to optimize where: laser beam (AOTF), gain, speed and concentration for both the probe and salt. They were systematically investigated for different levels according to table 5 and the gain was adjusted for each sample to get an intensity close to 150 in a small area if the image. The intensity was fixed to make gain the measured parameter. After this test was the intensity the measured parameter for all tests in the solutions.

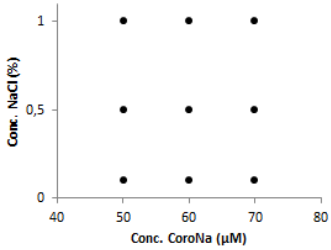
A test series according to fig 6a) was conducted where 5 combinations of salt/probe concentrations was evaluated, speed was changed from 200-800 Hz. AOTF was changed from 5-15% according to test 1 in tab 5. Gain values was set for each measurement to get a mean intensity of the picture around 150. This test was conducted once to get an indication of the interval for AOTF and probe concentration and to choose a speed. A second test series was made according to fig 6b) where 9 combinations of salt/probe concentration was evaluated. The AOTF for each combination was changed from 7-10% according to test 2 in tab 5, the test was made twice. These two tests were conducted to find a final concentration for the probe and the AOTF level to use for study changes in salt concentration.

Table 5: Evaluated levels for different parameters in salt solutions.

Parameter	Test 1	Test 2
C_{CoroNa} (μM)	20, 60, 100	50, 60, 70
AOTF (%)	5,7 9, 11, 13, 15	7, 8, 9, 10
Speed (Hz)	200, 400, 800	200
Gain	measured	measured
Intensity	150	150



((a)) Optimization for concentration range of CoroNa.



((b)) Optimization for final concentration of CoroNa.

Figure 6: Stages in optimize concentration range for salt and CoroNa. a) Samples was investigated for laser levels from 5-15%. b) Samples was investigated for laser levels from 7-10%.

The intensity changes in different depth of the sample and by time was investigated to find out how different conditions affect the result. The depth was studied in two replicates where the z-position was changed in steps of 10 μm from 10-100 μm under the cover glass in four positions in each sample. Time dependence was studied in 3 samples where 100 images was taken over 43 min and the change in intensity was noted. The specific setting for the replicates in the tests can be found in table 6. All replicates were made with concentrations of 60μM the probe and 0.5% for salt. Both the probe and salt solutions were investigated for auto fluorescence to find out if there were any background intensity that had to be taken into consideration.

The response in intensity with increased salt concentration was evaluated. Solutions according to series 2 in table 3 was prepared and tested. This was mainly done to find out if the intensity increased linearly in the interesting concentration interval. The test was made in triplicate with 10 measuring points in each sample and the specific settings can be found in table 6.

Gels: Both the objective, frame size, zoom and line average used was the same for the gels as for the solutions to enable comparison between the two systems. Speed was set to 200, AOTF was tested from 7-10%, the probe was used with the concentration 60μM and salt concentration 0.1 and 0.5% was investigated. Two replicates for salt concentration 0.1 and 0.5% with 4% gelatin and two replicates for salt concentration 0.5% with 8% gelatin was tested. The gain was changed to get a intensity of around 150 for AOTF 7,8,9,10% in four

Table 6: Fixed parameters used in tests to evaluate the intensity changes with depth, time and changed salt concentration.

Parameter	Depth	Time	C_{salt}
AOTF	8	10	8
Gain	594.7 and 608.1	568.3	610
Positions/replicate	4	1	10
Replicates	2	3	3

different positions in each gel. Gels with 1% and 0% of salt was studied without the probe solution evaluate the auto fluorescence in the gel.

Beside finding optimal setting another aim for the gels was to investigate different application methods for the prob. The four preparation methods from table 4 was studied over time to find out if the intensity changed over time. All samples were studied with AOTF 10 % and gain was set go get a mean intensity in the first frame around 150. The specifics for the time series can be found in table 7. The intensity change depending on the depth in the gel was investigated in similar way as for the solution. The experiment was made once in a gel with salt concentration 0.5%, gelatin concentration 4% and with mixing the probe into the gel solution. Both laser beam and gain were fixed to 7% and 580 respectively and the intensity was noted for changes in depth from 10 - 100 μm under the cover glass in steps of 10 μm in three different positions.

Table 7: Time dependence in gels

Parameter	Drop on gel 1	Drop on gel 2	Drop on gel 3	Mix in gel
Gain	660	733	772.9	670
Time	43 min	132 min	150 min	150 min
Frames	100	220	250	250

3.3 Preparation and method development for heterogeneous model systems

In the second part of the project, the heterogeneous model system dough and bread was studied. The dough was studied as a step between the homogeneous system and the real food product (bread). The changes from a simple to a complex system and how salt interacts with the different components in flour was evaluated.

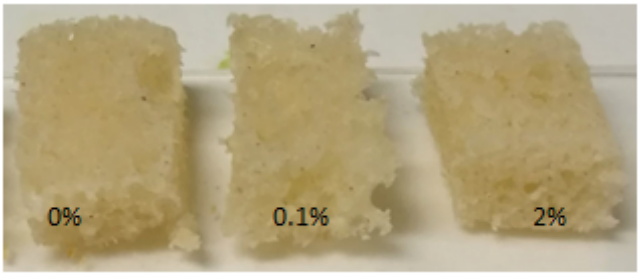
3.3.1 Preparation of dough and bread

Dough: The dough was prepared by adding salt and water on wheat flour weight basis [11]. The chosen ratio for flour:water was 10:6 and salt concentrations between 0.1-1% was tested with particle size of 0.1-0.3 mm. The final dough recipes can be found in table 8. All ingredients were mixed, and the dough was kneaded for a couple of minutes until it had a good texture. The doughs were kept in beakers covered with plastic foil before analysis to keep them from drying. To get a flat surface a small amount of the dough was placed in a metal cup. A cover glass was placed on top of the dough after the probe had been applied and dried. The cup was filled so the cover glass was attached to the top of the cup and touched the surface of the dough, see fig 7 a).

Table 8: Ingredients in tested dough.

Ingredient	Amount
Wheat flour	5-10 g
Water	60% of flour weight
Salt	0.1-1% of flour weight

((a)) Prepared sample of dough placed in a metal cup



((b)) Prepared samples of bread with three different salt concentrations, 0%, 0.1% and 2%. Each bread is approximately 1 cm wide

Figure 7: Prepared samples of dough and bread ready for analysis.

Bread: Bread with different salt concentrations was baked in the bakery at RISE. In total seven breads was baked with different salt content, water type and salt particle size. The recipes can be found in tab 9 where the yeast was mixed with water in a stock solution with either tap or distill water. For one bread was salt, yeast and water mixed together to make sure that the salt was dissolved before mixing with the flour. The dough was mixed for four minutes in room temperature before putting to proofing. Bread with no salt, 0.1% and 2% salt were prepared to study extreme conditions and large differences in the overall salt concentration. The breads with 0% salt and 0.1% and 2% in tap water was proofed for 60 min at 40°C and with relative humidity of 75%. The three breads with 2% salt and distill water was proofed for 60 min in 35°C and 85% relative humidity. All breads were baked for 18 min in 220°C during constant rotation. The breads were cut in slices and was stored in a

freezer before analysis. The bread was toughed wrapped in plastic foil to keep from drying. A small piece of the bread crumb was cut out with a sharp knife and placed on an objective glass. The probe was dropped on top of the bread, the finished sample can be seen in fig 7 b).

Table 9: Ingredients in tested bread with different salt concentration. index d indicates distill water and index t indicates tap water. Index 1 indicates salt pre-dissolved in water, 2 indicates small. salt particles and 3 indicates big salt particles.

Ingredient	**0%**$_d$	**0%**$_t$	**0.1%**$_t$	**2%**$_t$	**2%**$_{d,1}$	**2%**$_{d,2}$	**2%**$_{d,3}$
Wheat flour	94g	94g	93.8 g	90.7g	90.7g	90.7g	90.7g
Water	54g	54g	49.7g	49.8g	56g	54g	56.5g
Yeast	2g	2g	2g	2g	2g	2g	2g
Olive oil	-	-	4.5g	4.5g	-	-	-
Salt	-	-	0.3g	3g	3g	3g	3g

3.3.2 Development of a method for detection of salt content and distribution in heterogeneous systems

Dough: Due to the complex structure in dough compared to the homogeneous systems was the frame size, AOTF, objective and zoom adjusted. The settings and intervals that was changed can be found in table 10 and the probe was used in 30 and 40 μM. The effect of different settings was investigated in several steps to find a general combination that could be used for a specific area of interest. In the dough can it be interesting to study the distribution both in a large area as well as in a smaller area.

The probe was dropped on top of the dough, it would require either to high amount of the probe or to low amount of salt if the probe should be mixed in the dough with the wanted concentration. This decision resulted in diffusion of the probe and different incubation times was tested as shown in table 11. The system was thoroughly investigated for the relationship between intensity changes and the different parameters in the table. The incubation time is the time until the first image is taken from that the probe is dropped on the sample surface. The affinity was evaluated in samples of pure gluten and starch mixed with water and 1% salt. Intensity in the starch granules, gluten areas and the whole frame was investigating by study how the intensity changed over time.

The time dependence was evaluated by dropping the probe with a concentration of 30μM on the dough. Different incubation times and testing times was evaluated. Four replicates where prepared with salt concentration of 0.5%, laser beam 40%, 20x objective and zoom 1, the specifics can be found in table 12. The incubation time is the time from that the probe is applied to the sample to that the first image is taken. The total time is the time from the application to the last image.

Bleaching was evaluated in two replicates with one position in the first and three positions in

Table 10: Settings in dough that was evaluated and differed from the settings in homogeneous systems.

Setting	Test interval
Frame size	1024x1024
AOTF	25, 30, 40%
Gain	Kept under 700
Objective	10x (air) and 20x (water)
Zoom	1-4 times

Table 11: Parameters that was evaluated in dough and the used levels for each parameter.

Parameter	Test interval
Incubation time (min)	5, 8, 10, 15, 20, 30
Testing time (min)	10, 15, 35, 45, 60
Temperature (°C)	20, 40, 50, 60, 65
Affinity	pure gluten/starch
Bleaching	360 images in 4 min
Probe solvent	DMSO and ethanol

the second replicate. Totally 366 images were taken during 4 minutes with a salt concentration of 0.5% and probe concentration of 30μM. The settings were chosen to get as much laser light as possible on a very small area. AOTF 40%, frame size 512x512, 20x objective with 8 times zoom, speed 1000 and gain 691.1 was used.

Salt concentrations of 0.1, 0.5 and 1% was investigated in three replicates with different settings according to table 13. Images was taken over about 20 min for all samples and the intensity was noted. The different settings were tested to find the optimal combination of gain and laser for the dough samples in the interesting salt concentration interval.

Table 12: Settings for time dependence in four replicates.

Setting	1	2	3	4
Gain	597,6	624	637,2	689
Sample depth (μm)	30	20	28	25
Incubation time (min)	5	6	15	16
Total time (min)	41	66	62	77
Images	16	24	19	15

Table 13: Settings for concentration curve for 0.1, 0.5 and 1% salt in three replicates.

Setting	1	2	3
Gain	710.1	653.9	672.5
Offset	0.4	0.1	0.1
Sample depth(μm)	30	30	25
AOTF (%)	30	30	35
Incubation time (min)	8.5	3	6.5
Total time (min)	21	19	20
Images	6	6-7	6

To find the best way to apply the probe to the dough the stock solution of the probe was diluted in DMSO or ethanol and dropped on the dough. The incubation time was 7 min with DMSO and 6 min with ethanol. The cover glass was put on after 1 min, after the overflow of DMSO solution had been dried of and the ethanol had evaporated. The gain was higher in the test with DMSO due to much lower intensity, the specific settings is shown in table 14.

Table 14: Settings used when comparing DMSO and ethanol

Setting	**DMSO**	**ethanol**
AOTF	25%	25%
Gain	655.6	604
Sample depth	29μm	32μm
Incubation time	7min 30 sec	5 min 50 sec

The temperature dependence was evaluated in two replicates in dough with starch, water and 1% salt. 30μM probe deluded in ethanol was dropped on top of the sample and the cover glass was glued to the metal cup to prevent drying. A heating stage was used that raised the temperature with 2°C/min for the first test and 5°C/min for the second. The temperature was raised from 20°C to 65°C.

Optimization was done to evaluate the effect of different settings on the intensity. The evaluated settings where speed (S), incubation time (t_{in}), line average (l.a) and frame average (f.a). Three different values for each setting was tested and the total time was about 20 min for all testes, the specifics can be found in table 15.

Table 15: Evaluation of different settings effect on intensity changes over time in dough. The evaluated settings are: Speed (S), incubation time (t_{in}), line average (l.a) and frame average (f.a)

Setting	$\mathbf{S}_1$	$\mathbf{S}_2$	$\mathbf{S}_3$	$\mathbf{t}_{in,1}$	$\mathbf{t}_{in,2}$	$\mathbf{t}_{in,3}$	$\mathbf{l.a}_1$	$\mathbf{l.a}_2$	$\mathbf{l.a}_3$	$\mathbf{f.a}_1$	$\mathbf{f.a}_2$	$\mathbf{f.a}_3$
Speed	200	400	800	200	200	200	200	200	200	200	200	200
t_{in}(min)	6.3	6.6	7.5	10.6	20.2	29.75	9.7	11.1	12.2	7.8	7.5	7.5
l.a	8	8	8	8	8	8	1	4	8	1	1	1
f.a	1	1	1	1	1	1	1	1	1	1	4	8
Gain	614.3	645.4	658.9	613.1	575.6	506.4	608.1	618.4	622.5	590	575.6	506.4
t_{tot}(min)	21.8	20.75	21.75	26.5	35.6	44.1	21.4	21	21.45	20.5	20.5	20.5
Images	8	7	8	8	7	7	6	6	5	7	7	7

Auto fluorescence in dough with 1% salt was studied to find out if the dough itself gave any fluorescence in the studied conditions. The dough was studied with laser 42% and gain 838.2. To find out if the probe interacts with other components in the dough was dough without salt studied with 30μM probe deluded in ethanol, laser 25% and gain 645.

Bread: Bread has an inhomogeneous surface but the structure can still be visualized in a good way. The used technique for visualization was to take a stack of images in layers with a total depth of about 1 mm. The stack was used to create a maximum projection of the structure that gives a 3D experience. Different ranges in the z-direction was tested as well as

different intervals in the z-direction between each image in the stack.

The bread analysis has been made on two different microscopes, which gives some differences in the used settings. Leica confocal SP2 has been used to investigate different settings. Basic understanding of the structure and working procedure was gained and the investigated parameters can be found in table 16. Leica confocal SP5 was used to study bread with different salt content, particle size as well as large areas of bread with several projections put together. Effects like drying of the bread and diffusion of the probe was investigated by making projections over time at the same position. For Leica SP5 the same settings were used for all images to enable comparison, the specific values are shown in table 17.

Table 16: Studied parameters on SP2

Parameter	**Test interval**
AOTF	7,9,10,11%
Gain	500-600
Objective	5x and 10x
Zoom	1-4 times
Line average	1,2,4
Z-range	300-1500μm
Image distance	10, 20, 30, 40, 50, 75, 95, 145μm

Table 17: Settings for 3D imaging on SP5

Setting	**Value**
AOTF	8%
Gain	506
Image distance	50μm
Speed	400
Objective	5x
Line average	2

To compensate for background fluorescence from the probe a program was set up to remove the intensity from the probe in the other samples. The compensation was made in similar way as in Konitzer et al [12] where a mean value for the intensity of the reference samples without salt were subtracted from the other samples. All pixels with intensity lower than the mean intensity from the background fluoresces was subtracted from the image.

3.4 Statistical analysis

The calculations and data handling were made with Microsoft Excel. For evaluation of salt solutions and gels the measured gain values was normalized to intensity 150. When replicates where made mean values was used and the standard deviation was calculated. When changes in the intensity was investigated in time series the values was calculated as % changes from the first image to enable comparison between different replicates where the settings had been changed.

4 Results and Discussion

The results are divided into homogeneous and heterogeneous systems where the result for each part is presented after increased complexity of the system.

4.1 Determination of salt content in homogeneous systems

Homogeneous systems include salt solutions and gels. The effect of different settings was studied along with changes of physical parameters. Some settings and conditions have been constant for all samples. The used objective was a 20x water objective, the imaged was zoomed 4 times and line average 8 was used. Images were taken at a depth of 30μm under the cover glass except when the depth was evaluated. When replicates were made, the presented data show the mean values with the standard deviation. Gain level was investigated by finding the gain that gives an intensity in the studied area of around 150, ex in fig A1 in appendix. The measured gain level where normalized to represent the value that gives an intensity of exact 150. Changes in intensity for evaluation of time and depth were calculated as percentage change from the intensity in the first image.

4.1.1 Evaluation of intensity changes depending on salt concentration and changed settings on the microscope in liquid-based systems

The first step of the project was to evaluate the behavior of the probe in solutions with different salt concentrations. Different settings according to table 5 and figure 6 in the method was investigated to find out how they affect the result. A test according to fig 6 a) in the method with different speed, AOTF and five combinations of salt and probe concentration is shown in fig 8. The figure shows the required gain to get the intensity 150 in the studied area. The optimal gain range for the microscope is 500-700 to exclude effects on the image that might occur if extreme gain values are used. The intensity should be well distributed within the image and only a few completely black and with pixels are preferred. To get the intensity as even as possible in the studied area was a 20x objective and 4x zoom used. With low magnification does the corners and edges of the image become darker and that is reduced with the use of zoom. To reduce the effect completely was only a smaller area of the image studied. An image of a salt solution with the studied area marked can be seen in fig A1 in appendix. The intensity can only be measured after the image has been scanned so the gain was changed until an image with the mean intensity close to 150 was achieved. Before the data was analyzed all gain values were normalized to represent exact 150 in intensity.

The results in fig 8 show that the needed gain rises with increased speed and decreased laser. The best speed for all combinations A-E is 200 Hz, which had most results within the preferred gain range. Low AOTF decreases the risk for affecting the sample and probe by bleaching. The goal was to be able to keep the AOTF under 10% and the result indicates that the optimal AOTF is between 7-11% for almost all combinations when the speed is set to 200 Hz. Comparing the probe concentrations 20μM (A-B), 60μM (C) and 100 μM (D-E) indicates that the optimal probe concentration is around 60μM. 20μM require to high laser or speed to end up in the preferred gain interval and 100μM is unnecessarily high concentration, especially for 1% salt.

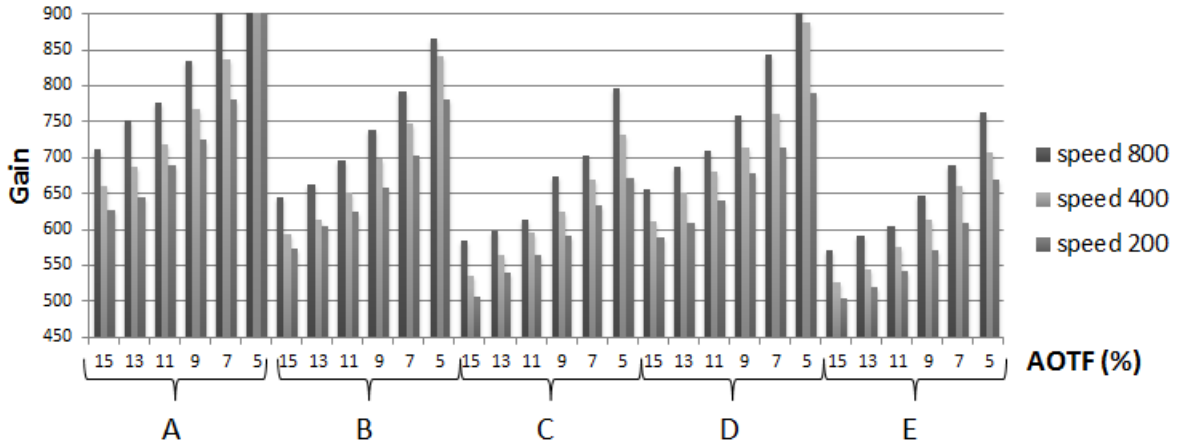


Figure 8: Gain levels where the speed differed between 800 Hz, 400 Hz and 200 Hz and AOTF from 5-15% was tested for different combinations for concentration of CoroNa and salt. A=20μM CoroNa and 0.1% salt, B=20μM CoroNa and 1% salt, C=60μM CoroNa and 0.5% salt, D=100μM CoroNa and 0.1% salt, E=100μM CoroNa and 1% salt

A test series according to fig 6 b) in the method was conducted by use of the results from fig 8 to find the optimal AOTF and probe concentration. AOTF from 7-10% and probe concentration from 50-70μM was evaluated for salt concentrations 0.1, 0.5 and 1%. The result is presented in fig 9 with mean values and standard deviation from two replicates. Like in fig 8 the gain was normalized to mean intensity of 150 in the studied area.

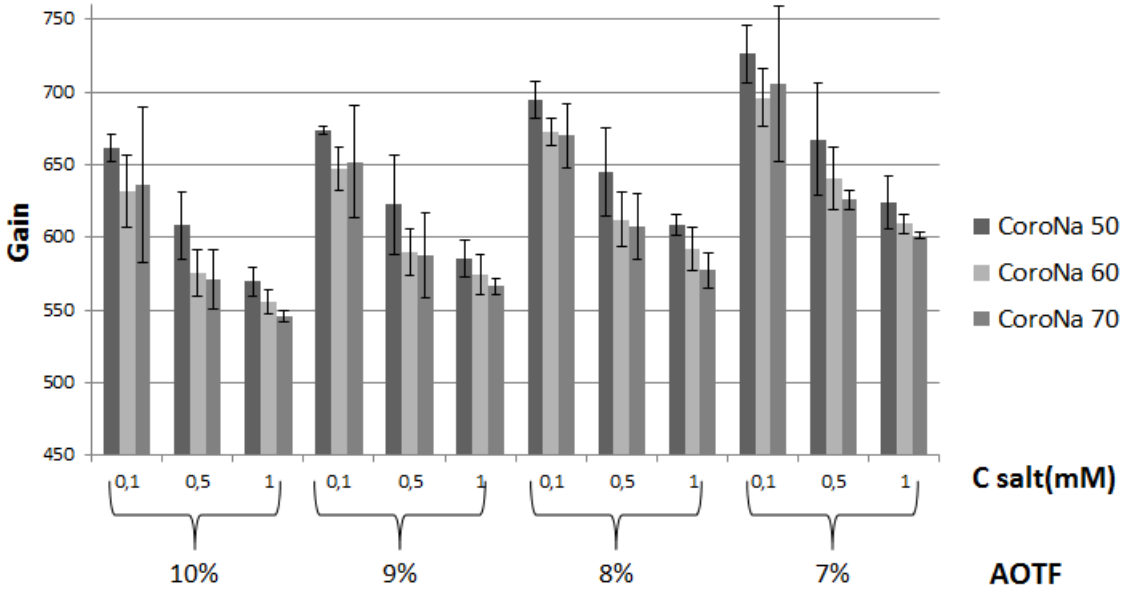


Figure 9: Gain levels where the AOTF was changed between 7-10% and the concentration of the salt varied between 0,1-1% and the CoroNa concentration varied from 50-70μM. Mean values for two replicates and standard deviation are presented.

The result shows a trend in the behavior of the system. The gain increases for decreasing salt

concentration for all series and AOTF levels. Lower laser and probe concentration requires higher gain. The system seems to behave linear both with changed laser and for the chosen salt concentration interval. Both the laser and probe concentration would preferably be kept as low as possible to eliminate risk of affecting the outcome of the result. With the result from fig 9 the probe concentration 60μM and AOTF 8-9% is optimal to keep the gain in a good range. The standard deviation is high for a few concentrations, especially for probe conc 70 μM and salt conc 0.1%. The variation in intensity for the low salt concentration can be explained by the low intensity and that the error increases with the dilution. There is an overlap for the standard deviation between the different tests. The overlap indicates that the variation between different samples has an influence on the result that has to be taken into consideration.

4.1.2 Effects on the intensity based on changed salt concentration, sample depth and time in liquid-based systems

With the results from fig 9 a test was conducted to evaluate the intensity change for fixed settings and changed salt concentration from 0.05-1%. The settings are found in table 6 in the method and three replicates where made with 10 measuring points in every sample. The result shows the mean intensity for all measuring points of each concentration. The result is presented in fig 10 and shows a linear increase in intensity with increased salt concentration.

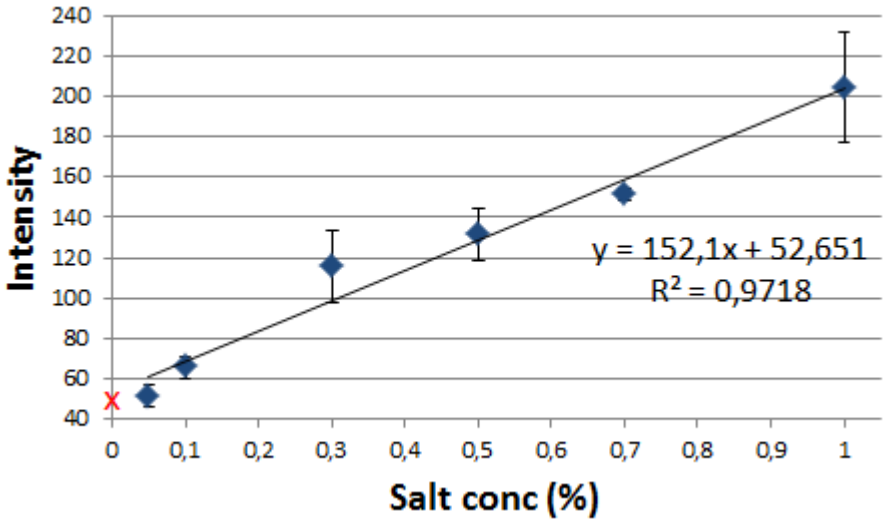


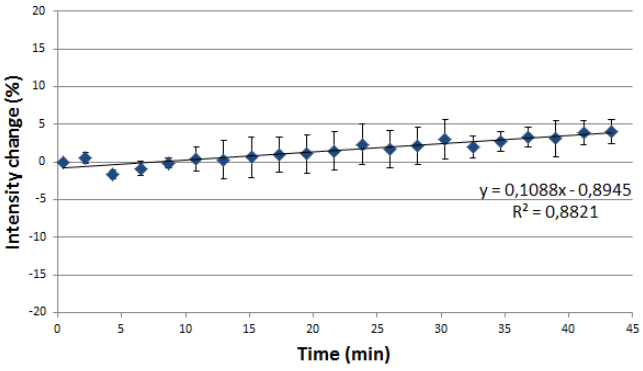
Figure 10: Measured intensity for salt concentrations from 0.05% - 1%. Concentration of CoroNa was 60μM, AOTF 8%, gain 610. Mean values for three replicates with 10 positions/replicate and standard deviation.

Compared to previous results from Martin et.al [44] and Iamshanova et.al [42], this result differ in the range that the intensity increases linearly. Their results indicate that the intensity follows a logarithmic curve with a linear range from 0-100 mM [44] and 1-60 mM [42] salt respectively. Recalculated to mM salt is the concentration range from fig 10 8.5-170 mM. The studied solution differs between the studies and in this project the salt was diluted in distilled water. In the two articles are either a choline solution with and without addition of potassium

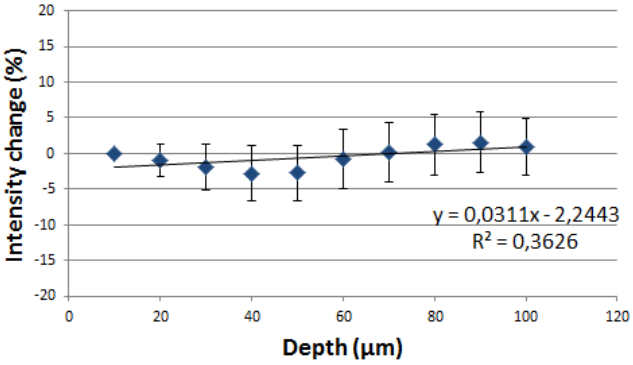
ions [42] or MOPS with addition of potassium ions used [44]. Similar to the previous results does fig 10 show a background fluorescence with no salt in the sample, red "x" on y-axis in fig 10. This indicates an auto fluorescence from the probe that has to be taken into account if the sodium ion concentration shall be estimated. The background fluorescence is about 50 on the intensity curve that is marked on the y-axis.

To investigate the intensity changes over time images where taken continuously over a 43 min period in three replicates. The AOTF was set to 10% which is higher than the laser used for the test with different salt concentrations. That was to eliminate the possibility for an effect on the intensity in the concentration test due to bleaching. The result in fig 11 a) show the mean intensity for every fifth image in the series and the total series contained 100 images. The result indicates a slight increase in intensity over time, but the increase is not significant due to the standard deviation. It seems like the signal is more stable in the beginning by comparing the size of the standard deviation. The result indicates that the probe is rather stable over time and that as long as the distribution is homogeneous, and since the system is relatively stable the signal will have low variation. That the variation is towards an increase in intensity indicates that the laser does not affect the probe in a negative way.

Another factor that could influence the result is if the intensity changes with the depth of the sample. Four different positions in two samples was studied at different depths from 10-100μm and the result can be found in fig 11 b). The result indicates that there could be small changes in the intensity depending on the depth, but the changes are not significant. Similar to the time test in fig 11 a), the signal has low influence from the depth in the sample as long as there are no other disturbance.



((a)) Intensity changes over time. Mean values for three replicates.



((b)) Intensity changes with depth of the sample. Mean values for two replicates with four positions/replicate.

Figure 11: Investigation of the effect of time and depth in sample on the intensity. All samples were made with salt concentration of 0.5%, probe concentration of 60μM. The laser was set to 10% for the time test and 8% for the depth test.

The behavior of the probe in salt solutions seems to be linear accounting to the different factors that has been evaluated. The probe also responds linearly to change in the microscopic settings and gives no sing of bleaching when images are taken at the same position over long time. The fact that the probe itself is stable makes it easier to evaluate more complex systems where the sample and surrounding can influence the result. Since the probe gives some auto fluorescence it is important to have that in mind when other samples are investigated.

4.1.3 Optimal setting and important factors to keep in mind for studies in liquid-based systems

To evaluate salt concentrations up to 1% in water solutions on the Leica SP2 is the following method preferred:

- Mix equal amount of salt solution and probe solution in a small metal cup with a 1 ml pipette. Put a cover glass on top of the cup and use probe concentration of 60μM.
- To avoid shadowing effect form the outline of the image, use 20x objective, 4x zoom and measure intensity in a smaller area of the same size in all samples.
- The following settings are preferred: Line average 8, Speed 200, AOTF 8%, gain around 600
- Make sure that samples without salt are studied and that the intensity increase is linear in the studied salt concentration interval. In this way it is possible to use the created curve with increasing salt concentration for calibration.
- The solution has low effect from studies of different depths and times
- The probe detects sodium ions, which means that the salt has to be dissolved to be detected and that the sodium doesn't bind to something else in the solution.

This method is optimized for one specific microscope and might need changes if used on another microscope or with changed conditions. If a wider range in salt concentration is studied it is important to make sure that the intensity increases linearly in the whole concentration interval, see fig 4 in the theory for effects on to high sodium concentration.

4.1.4 Effects of salt content and settings on the CLSM in a semisolid homogeneous system

To find out how different probe application methods affect the sample was two methods tested according to table 4 and 7 in the method. Gels with 4% gelatin and 0.5% salt was prepared by either dropping the probe on the gel or mixing it into the gel. Fig 12 shows the mean values of the intensity change vs time for two replicates of both homogeneous and inhomogeneous probe distribution. The intensity change is based on image number and for the inhomogeneous samples was the first 100 images used due to different number of images in the two series. This test was done to find out how much the intensity is affected by inhomogeneous application of the probe and if the intensity stays stable over time as in the solutions.

Fig 12 shows that with an inhomogeneous probe distribution, orange curve, the intensity decreases logarithmic and does not stabilize within the testes time range that is approximately 45 min. For homogeneous probe distribution, blue curve, the intensity does not decrease as in the inhomogeneous distribution. The signal is not stable as in the solutions and the intensity increases with about 10% in total. The signal seems to stabilize after about 200 frames, which is two hours after the test started, which makes the total time from adding the probe to a stable signal around three hours. Even if the homogeneous distribution gave an unstable signal the changes in intensity was lower than for the inhomogeneous distribution. After the first test, all gels were prepared with homogeneous distribution of the probe.

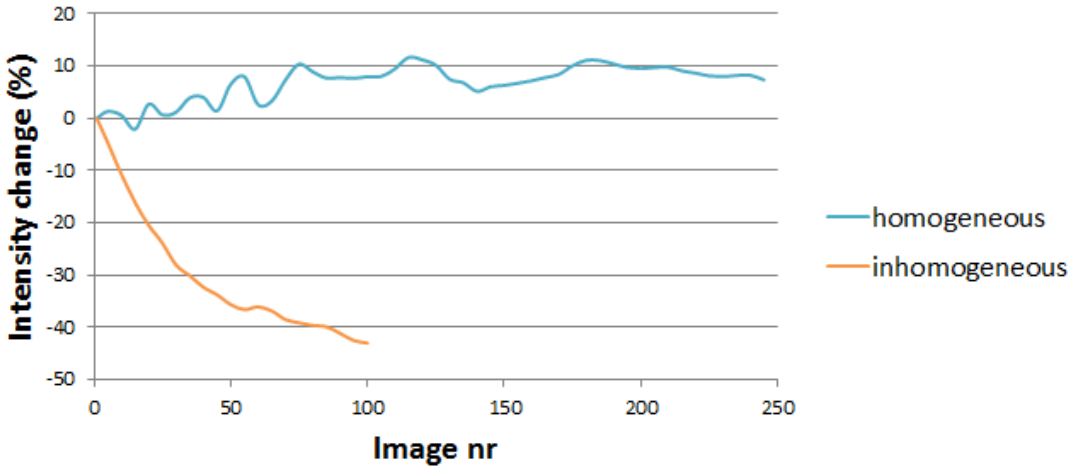
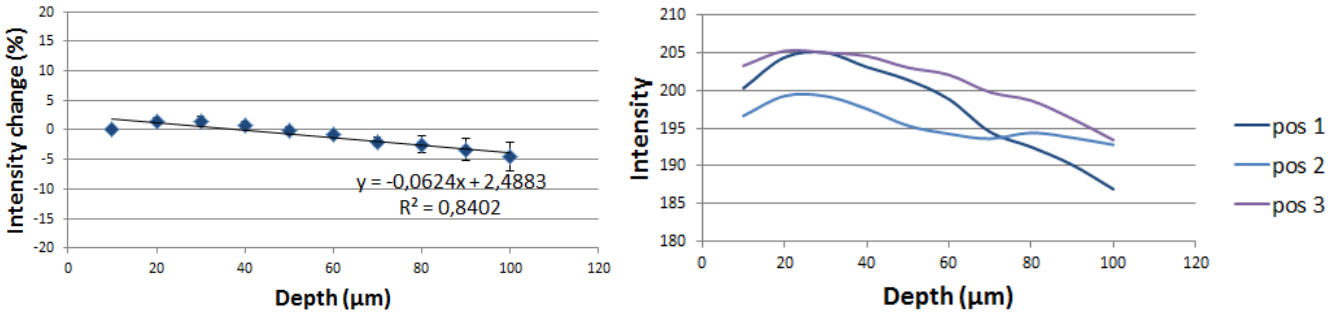


Figure 12: Time series of gels with two different probe applications: drop the probe on top and test after 20 min, or homogeneous distribution of probe in sample. Mean values for two replicates/application method.

A test for intensity evaluation in different depth of the gel was calculated similar to the salt solutions. The result is presented in fig 13 where a) shows the mean values of the intensity changes vs depth. Three different positions in one gel sample were studied and the depth is changed from 10 to 100 μm under the cover glass. The intensity decreases with a few percent, bigger change than in the solution (compare to fig 11), but the standard deviation is lower in the gel which makes the results more reliable. Fig 13 b) shows the intensity change in each position of the gel. The laser and gain was kept constant throughout the whole test which makes it possible to note the difference in intensity for the different positions. From the figure it is possible to see the tendency of instability that the time test shows in fig 12 and also that the change with depth between the positions differs. That the intensity decreases with the depth could be due to the gelatin that could influence the signal and make it weaker further down the sample.



((a)) Mean intensity change for different depth. Mean values for three positions and standard deviation.

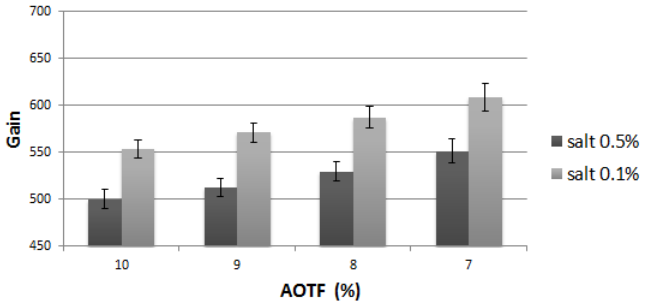
((b)) Intensity for each tested position

Figure 13

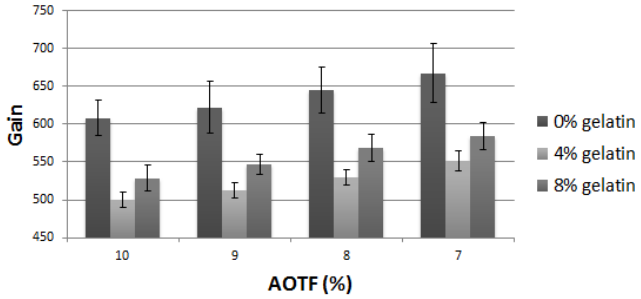
To find out how the system behaves with different concentrations of salt and gelatin was two

different tests conducted. In the first test the needed gain was noted for different AOTF for gels containing 0.1 and 0.5% salt and 4% gelatin, shown in fig 14 a). The figure shows the same behavior as the solutions where a higher gain is needed to reach intensity 150 for gels with lower salt content and the gain increases with decreased laser. The signals between the different concentrations are significant for the two replicates for all AOTF. The difference between the AOTF for each concentration is not significant, but the result indicates an increase of gain for decreased laser.

Two gel concentrations were tested, 4 and 8%, and was compared to the salt solution as shown in fig 14 b). Compared to the salt solutions, both of the gel concentrations required less gain. This could be due to that the used gelatin could contain some salt. But if the gelatin itself contain salt the gel with more gelatin would require lower gain. The gelatin and salt was added on water basis which resulted in a small difference in the salt content compared to the total volume. This could explain why the two gelatin concentrations gives different results.



((a)) Comparison of gels with 0.1 and 0.5% salt with 4% gelatin. Mean values for two replicates and standard deviation.



((b)) Comparison of gels with 4 and 8% gelatin with 0.5% salt. Mean values for two replicates and standard deviation.

Figure 14: The effect of changed salt concentration and gelatin concentration for AOTF form 7-10%. The sample with without gelatin is a salt solution

One may speculate that there is something else that is happening in the gels. The probe binds to free sodium ions which means that the isoelectric point may influence. If the isoelectric point is decreased, there are more free ions in the sample. Since neither the isoelectric point nor pH was evaluated there is a chance that the sodium ions bind to the gelatin after a certain concentration. The structure and transparency in the samples may also affect the outcome by changing the mobility for the probe.

For all tests except the depth was four different positions in each gel tested with AOTF 7-10% which gave a total testing time around 15-30 min. The gel structure seamed to change during the testing time with dissolution of the gel in some parts. This indicates that the gels might be unevenly mixed when the probe was added to the gel solution. To get a gel that is better mixed, larger volumes could be used to enable proper mixing in a beaker, but that would require larger quantities of the probe solution. The dissolution could also be an effect from the laser, indicating that the gel is sensitive to reheating. To overcome effects from the laser it is important to use as low AOTF as possible and to do the tests quickly and at different positions in the sample.

4.1.5 Optimal settings and important factors when semisolid systems are studied

The settings for gelatin gels could be kept rather similar to the solutions. The gain levels for the same salt concentration in a solution compared to a gel differs a bit, but both systems end up within the preferred gain interval when intensity is fixed to 150. The optimal preparation and working method for a semi solid system studied with the Leica SP2 is stated below.

- The probe concentration is connected to the intensity, which means that for samples with unknown salt concentration is it important to know the probe concentration. Use homogeneous probe distribution and the best result is achieved with a probe concentration of 60μM.
- Prepare the gel solution separately in a beaker. Add equal amount of gel solution and probe solution to a small metal cup with a 1 ml pipette and let dry for about 40 min. Put a cover glass on top and study with a 20x objective and 4x zoom.
- The following settings are preferred: Line average 8, speed 200 Hz, AOTF 7%, gain a bit under 600
- The time and depth have more influence in the gels then for solutions. Make sure that images are taken at constant depth and within similar time frame to exclude change in intensity.

4.2 Determination of salt content in a heterogeneous systems

The heterogeneous systems studied were dough and bread. Changes in the system behavior compared to the homogeneous systems were evaluated along with thoroughly investigation of information that can be obtained for the product. For this systems was 30μM of the probe used as in the article from Noort et al [11] instead of 60μM that was used for the homogeneous systems. This change was made to adapt the method to previous used probe concentrations. The studies in homogeneous samples show no effect of the fluorescent capacity over time. This indicates that higher AOTF could be used without risk for bleaching of the probe.

4.2.1 Basic behavior of a dough system to study affinity and dependence of time and salt concentration with CoroNa diluted in DMSO

The decision to decrease the probe concentration to match previous studies [11] resulted in a need to increase in AOTF compared to the homogeneous systems. The dough differed a lot in structure compared to the gels and solutions and the network of gluten and starch granules influences the signal and salt distribution. To find out the affinity of salt to starch and gluten mixtures with pure gluten or starch was studied.

Figure 15 a) and b) show the salt distribution in the pure samples of gluten and starch. In 15 a) the salt is evenly distributed, the different areas come from that the sample has a gum-like texture that was hard to mix evenly and to get a clean cut for the tested sample. 15 b) shows how the salt distributes around the starch granules and it seems like the concentration is more intense close to the surface of the granules. The black areas are inside the granule where the probe cannot access. 15 c) shows a dough with wheat flour, salt and water and compared

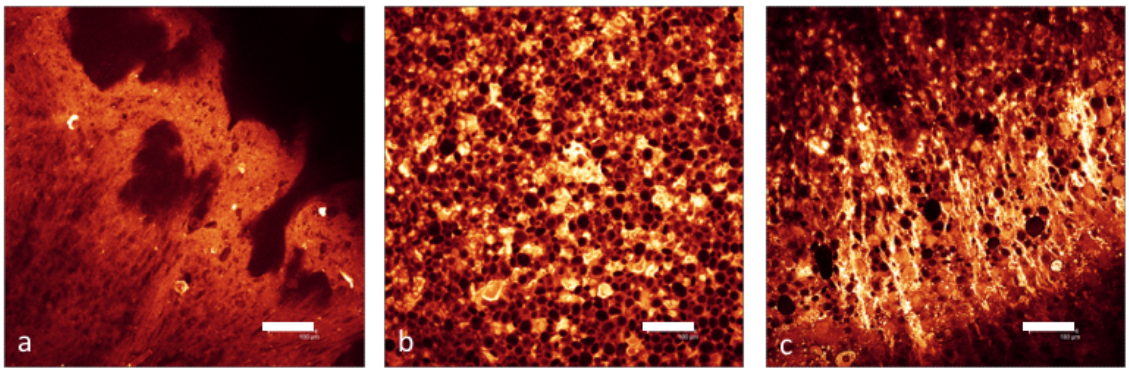


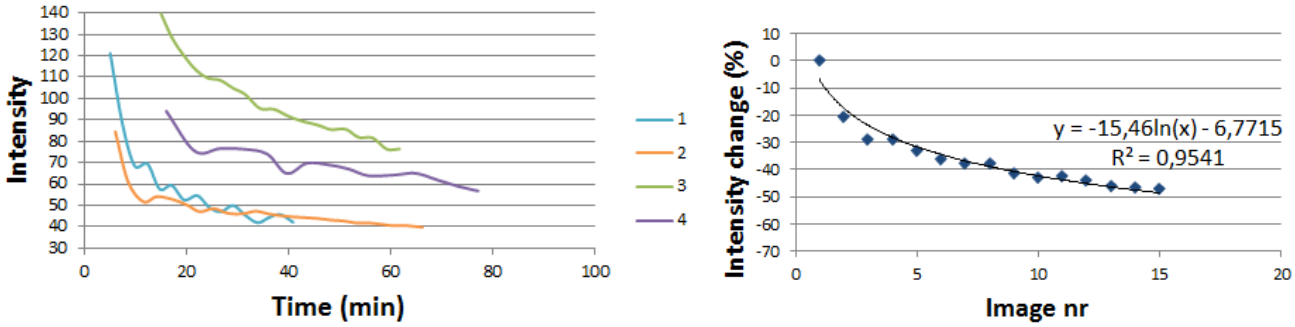
Figure 15: Salt distribution in mixtures with pure gluten and starch with 0.5% salt compared to distribution in dough with wheat flour. The white line represents 100 μm.

to the images in a) and b) it is possible to distinguish the starch granules as the black spots while the gluten network are the bright areas.

The three images in 15 a-c shows the complexity of the system that a dough provides, and the influence of the starch and gluten is important for the intensity of the image. This means that the intensity of samples with the same amount of salt can differ depending on the structure of the gluten network. The intensity can also differ within a sample depending on how the network formation looks like. Since the salt concentration seems to be higher in gluten rich areas, the salt will not be evenly distributed between the different components. The affinity of salt in the gluten is in line with the fact that salt has an influence on the formation of the gluten network as previous mentioned [8, 9, 19].

The tests in gels (fig 12) showed a decrease in intensity when the probe was applied on top of the gel. For the dough that method had to be used anyway because it would require to high quantities of the probe if it should be mixed in the dough. The intensity change over time was investigated with both different incubation times for the probe and different testing times as mentioned in table 11. The test was made to find out if the intensity stabilizes and how much influence the incubation time has on the intensity. The test was set up according to tab 12 in the method.

Fig 16 a) shows the intensity over time for the four replicates. All replicates show large drop in the beginning of the testing period and the intensity seems to stabilize a bit after 40-60 min especially for sample 2 and 4. Even if the signal stabilizes after some time, all sample has a rather large drop of intensity in the first few frames. The intensity drop is clearly shown in fig 16 b) where the mean intensity change per image for the four samples are shown. The graph shows a drop of 20% between the first and second frame and almost 10% between the second and third. After that, the decrease rate reduces but keeps decreasing throughout the whole test. The corresponding images for all series and curves where the intensity has been measured in gluten and starch regions can be found in fig A3-A5 in appendix. The intensity difference in the first three frames is clearly shown in the images for all series and also in the graph in fig 16 a). The decrease is also noticeable when study the starch granules and gluten areas separately, but it differs between the series. When the gluten network is followed is the decrease in intensity more constant over the whole test period and does not follow the same logarithmic form as in the whole frame and starch granules.

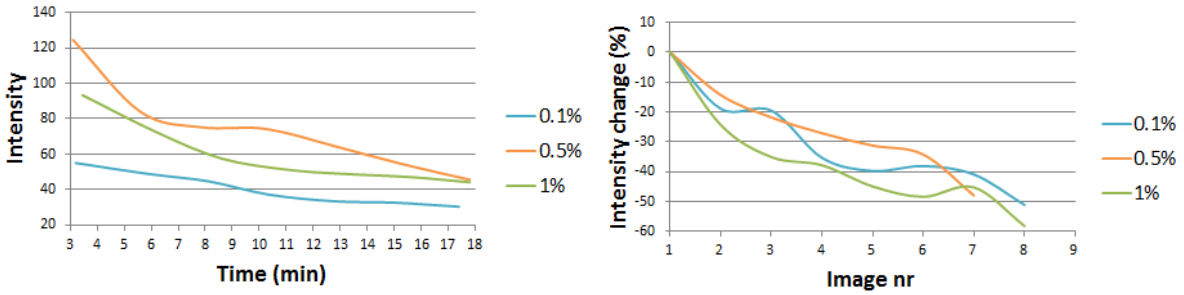


((a)) Intensity over time for 4 dough replicates with different incubation times of the probe.

((b)) Mean intensity change from the series in a)

Figure 16: Intensity over time in dough for four dough replicates with salt concentration 0.5%.

Three different concentration series where made according to table 13 for 0.1, 0.5 and 1% salt. To take into account the intensity change was images taken over about 15 min and all concentrations in the same series had similar incubation time. The series differed in settings and incubation time to evaluate the optimal settings. The results from the test is presented in fig 17 and the intensity was measured in the whole frame



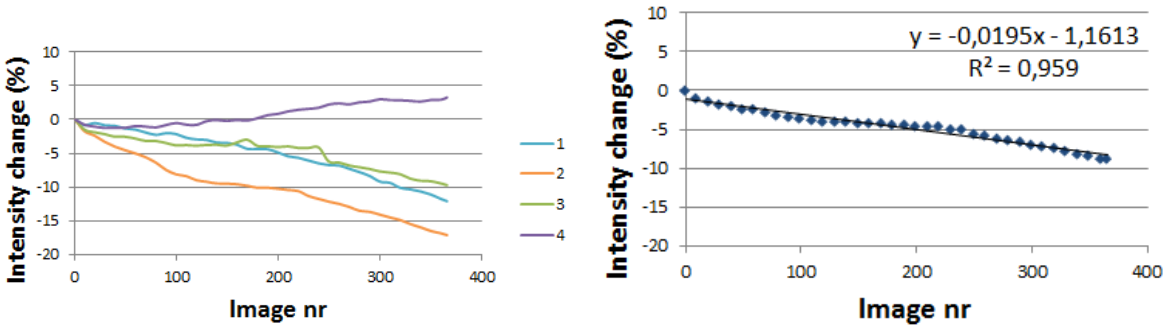
((a)) Intensity for salt concentration curves, with images taken over 15 min, one of three replicates

((b)) Mean intensity change by image number for all three replicates

Figure 17: Intensity changes over time for dough with salt concentration of 0.1%, 0.5% and 1%.

Fig 17 a) shows the intensity over time for one of the three replicates. The corresponding images for the test series in fig 17 a) and the graphs for the other two concentration series can be found in A6-A8 in appendix. The graph shows a difference in intensity between 0.1% salt and the other two concentrations. For the higher concentrations are the intensities flipped so the sample with 0.5% show higher intensity the 1%. This is the case for all replicates, the sample with 0.1% is a bit under the others that are more tight together but with flipped intensities to what is expected. The different replicates come from the same dough so there is a possibility that the doughs have been mixed up, although they have been thoroughly marked. Fig 17 b) shows the mean intensity change for the three replicates based on image number. The same tendency as previous tests with a rather big decrease in the first few frames can be seen. The intensity drop is also equivalent for all concentrations indicating that it is independent of the salt concentration.

To further investigate the noted intensity drop a test for bleaching of the probe was set up. The single purpose of this test was to find out if the probe is affected by bleaching with the laser. The image quality was not important, so both the frame size and line average were decreased, and the scan rate increased to be able to increase the number of scans for a short period of time. The zoom was also increased to keep the area in focus as small as possible. The bleaching was made four times over four minutes and 366 images was taken. Fig 18 a) shows the intensity change for each of the four replicates. Replicate 1 was made in a separate sample and replicate 2-4 are from different positions in the same sample. The graph shows a decrease in intensity in three out of the four replicates and an increase in the last one. The mean intensity change can be seen in fig 18 b) that indicates a drop of almost 10% in total. Since there is no drastic drop in the intensity it seems like the probe does not bleach. The fact that the intensity still decreases might be due to diffusion of the probe into the dough.



((a)) Bleaching in dough over four min

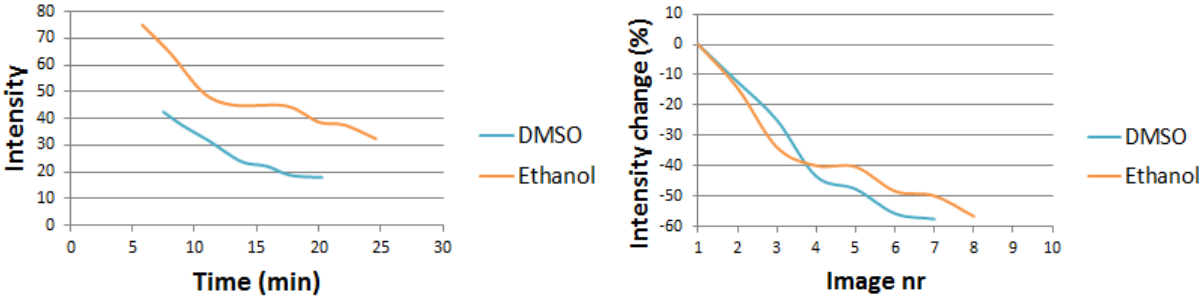
((b)) Mean bleaching of replicates in a)

Figure 18

4.2.2 Thorough evaluation of factors that may influence the intensity and optimization of the dough system with use of CoroNa diluted in ethanol

DMSO, that has been used for dissolving the probe in the homogeneous systems does not evaporate in room temperature and it does not sink into the dough. Instead ethanol was considered as diluting agent since it evaporates in room temperature and will leave the probe in the top layer of the sample. A comparison of the intensity and intensity change over time between the two dilution methods is shown in fig 19 a) and b) and the specific settings can be found in table 14. 19 a) shows that the dough with ethanol has higher intensity than the dough with DMSO even if lower gain is used. 19 b) shows the intensity drop with the number of images for the two samples, with a bit more drop for the DMSO-sample in the end of the test. The result shows that there will be a drop in the intensity independent of what diluting agent that is used. The intensity will be higher if ethanol is used which will result in lower AOTF. Further on will all samples use the probe diluted in ethanol.

Some other factors that might affect the intensity is the line and frame average, the scan speed and the incubation time. Three different levels of these parameters where tested according to table 15. Both the averages and the scan speed affect the amount of laser that the sample is exposed to. Even if the bleaching test shows that the probe itself is not affected by the laser, it is good to exclude the possibility for them to affect the result. Incubation time is the

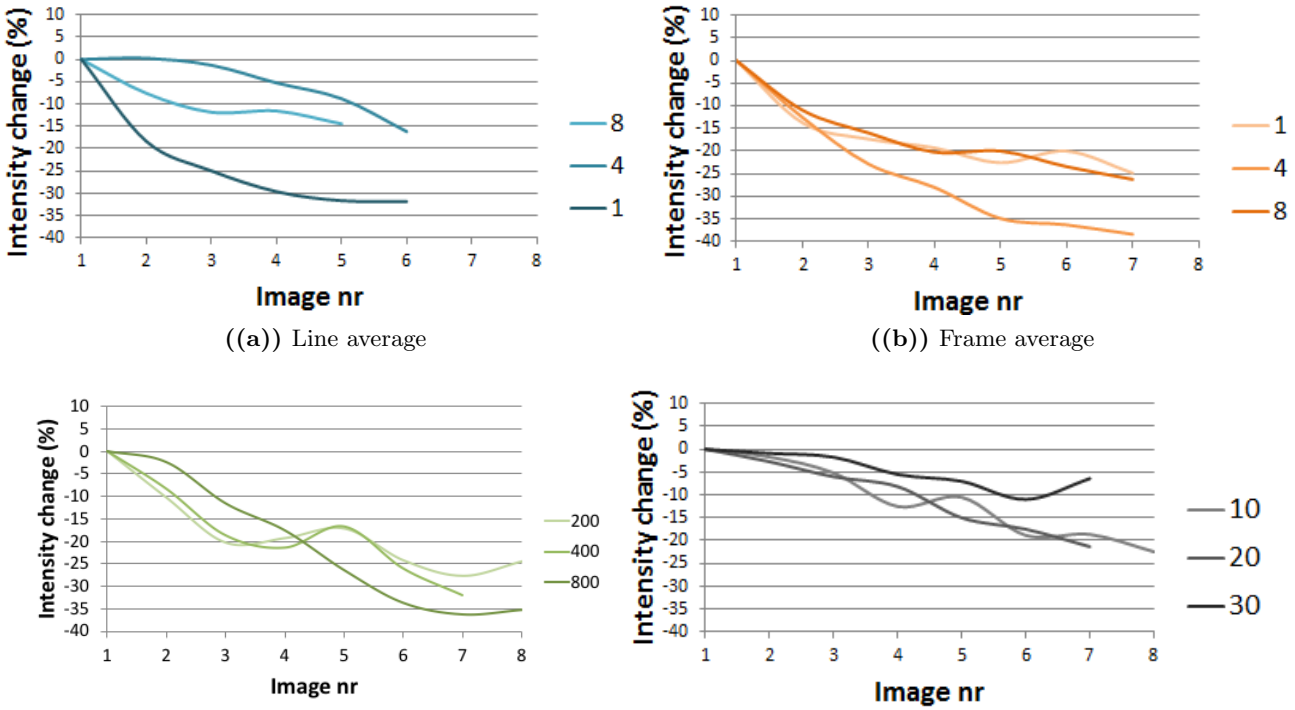


((a)) Intensity in dough with probe deluded in DMSO and ethanol

((b)) Intensity change per image for probe with DMSO and ethanol

Figure 19

most likely source for the intensity drop and has so far been tested with time up to 15 min. Incubation times of 10, 20 and 30 min was tested.



((a)) Line average

((b)) Frame average

((c)) Scan speed (Hz)

((d)) Incubation time (min)

Figure 20: Intensity change as an effect of changing values for certain parameters

Fig 20 a-c shows the effect of line/frame average and scan speed for one replicate of each test. The result shows a rather drastic drop of the intensity in the first images for all tested levels except line average 4 in 20 a). This could be a coincidence since both line average 1 and 8 shows a greater drop. Fig 20 d) shows the effect of incubation time and the drop for all of the tested times is lower than in any of the other tests, 5-20% in impact compared to 20-40%.

That the curve with least change in intensity is the one with longest incubation time is in line with the theory that the intensity drop is connected to the incubation time. Worth noticing is that the sample with line average 8 in 20 a) and incubation time 10 min in d) has very similar settings and only differs with 10 in gain and 2 min in incubation time. The intensity drop for line average 1 more the 10% in the first three frames in 20 a) and only 5% for incubation time 10 min in 20 d).

To be able to use the method in a sample with unknown concentration or inhomogeneous salt distribution it is important to know how much background signal that has to be reduced to get the correct intensity. Both the auto fluorescence in dough without the probe and a dough without salt but with the probe was analyzed. The dough without probe gave very low signal at extreme laser and gain (42% and 838.2) but the dough without salt but with the probe showed similar structures as the ones with salt and probe but with lower intensity. A comparison between a dough without salt and with 0.5% salt can be seen in fig 21 where the image is cropped to highlight the structure. Both doughs were stained with 30μM of the probe deluded in ethanol and images were taken with laser beam 25%.

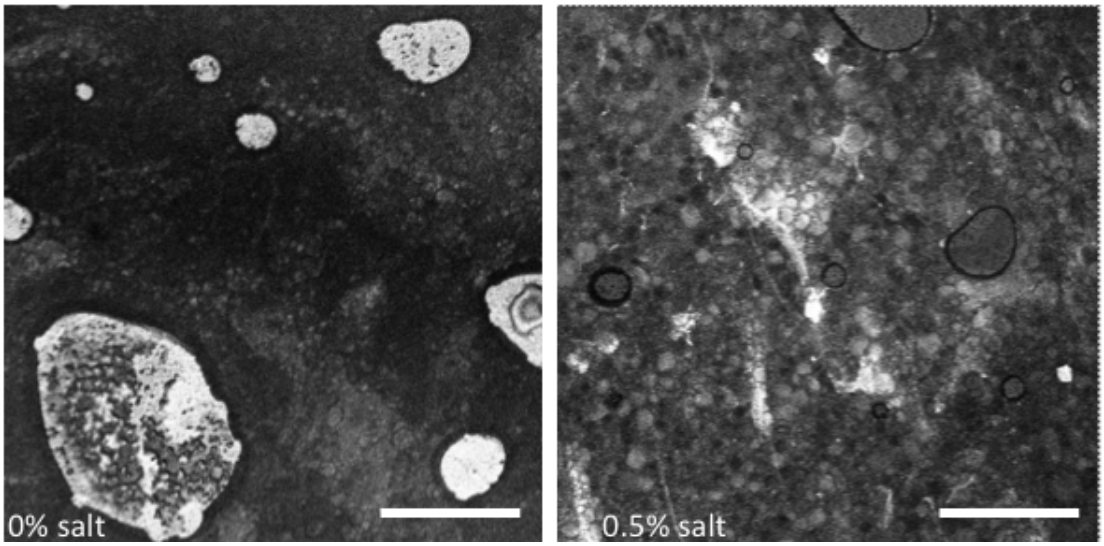


Figure 21: 0% salt with gain 645 and 0.5% salt with gain 643.3. Both images taken with AOTF 25%. The white lines represent 200 μm.

In fig 21 a) it is possible to see starch granules and the gluten network as the brighter area. There is also some bright bubbles that could be areas with water that the probe is attracted to. In fig 21 b) all structures are more visible, and the gluten network is shown as bright lines. Small bubbles can be seen, even though they are darker indicating that there is higher salt concentration elsewhere that attracts the probe. To get the true intensity for the salt distribution in fig 21 b) the intensity for a sample with the same settings and no salt have to be excluded. This can be made by imaging like in Konitzer et al [12]. There was the mean intensity from images without salt removed from the other images. In this way, only the areas with higher intensity than the background intensity were displayed. Since dough only was considered as a model system, no imaging was done to compensate for the background fluorescence. If further studies will be made on salt distribution in dough does a proper method to reduce the background fluorescence has to be developed. The method

used by Konitzer et al works for bread where the distribution seems homogeneous considering different components. In dough does the reference with no salt show differences in affinity between components. This can give false results if the probe itself is more attracted to e.g. gluten but the salt is equal distributed between gluten and starch.

4.2.3 Possibilities to study changes in salt distribution during heating and with different magnification as well as inhomogeneous salt distribution

To find out how the salt moves when the starch granules are swelling would it be interesting to follow the swelling process live by heating the dough during analysis. By use of a heating stage was a starch sample with 1% of salt and the probe heated to find out if the probe could stand the temperature increase and if the swelling of starch granules could be followed. Two replicates where tested, but it was not possible to heat the sample to more then 65°C before the signal quickly faded and disappeared. This indicates that the probe is not functional in high temperatures and can not be used to investigate swelling of starch granules at higher temperature than 65°C.

Salt distribution can be studied at different length scales in the sample to analyze and compare the distribution both close to the starch granules and in a wide area in the sample. By study the distribution with higher magnification it is possible to evaluate the affinity of salt to different components of the dough. Although previous studies conclude that the distribution in bread is even [12], the imaging from dough suggest otherwise. By using different magnification would it be possible to study the affinity closely. In fig 22 the magnification for the same position in a dough sample has been change from 1 to 2 to 4 times with a 20x objective. The result gives an indication of how the focus can be changed from a wide area with zoom 1 to close-up investigation of some specific granules with zoom 4.

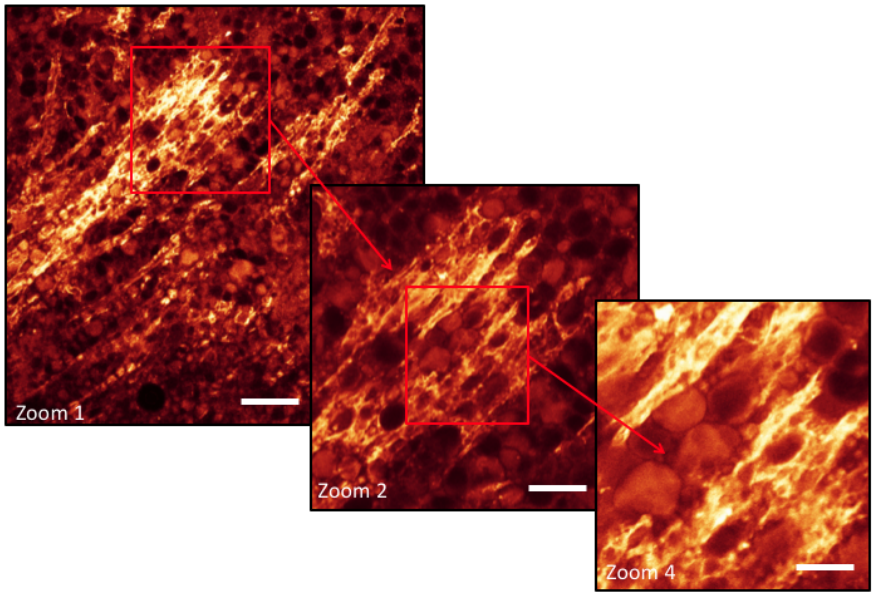
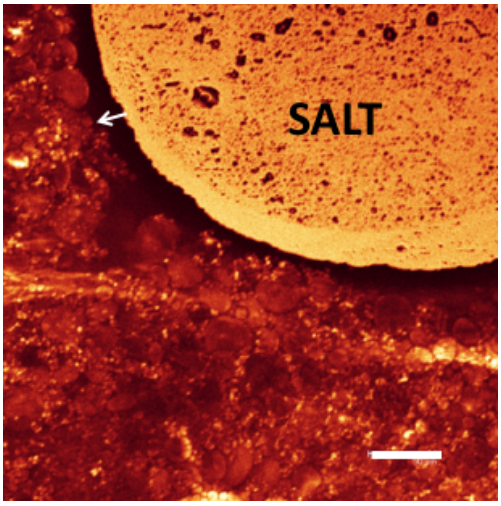
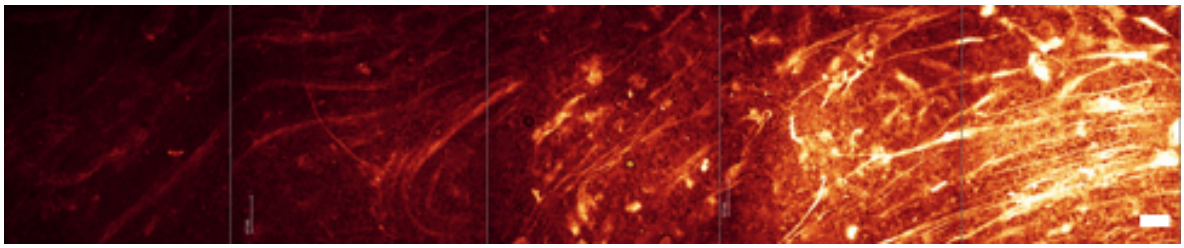


Figure 22: Example of different magnification in the same area of the dough. The white lines represent 100, 50 and 25 μm.

When large salt particles were mixed into the dough it was not possible to find the particles if they where not at the very surface of the sample. Even at the surface it was hard to distinguish if it was the salt particle that had been found, example in 23 a). The images show what seems to be a salt particle in to the edge between the particle and the dough. The images show that there is an empty space between the particle and the dough, marked with an arrow, which may be due to that the salt particle has been pushed down into the dough. Inhomogeneous distribution was also studied by mixing of two doughs with different salt concentration, image shown in fig 23 b) where a series of images was taken in a row to capture the overlap between the two concentrations. The result shows that the side with high salt concentration is brighter than the side with lower salt concentration. This indicates that the method will be able to detect different concentrations within a sample.



((a)) Big salt particle in dough. The white line represents 50 μm.



((b)) Images taken over bigger area with low salt conc on the left side and high salt conc on the right side. The white line in the bottom right corner represents 200 μm.

Figure 23: Intensity change as an effect of changing values for certain parameters.

4.2.4 Limitations and problems for the dough system compared to homogeneous systems and the best method for studies in dough

Compared to the homogeneous systems, the dough is more complex to study and to get reliable data from. The problem is that there are rather big variation over time and for different replicates. To compare different samples, it is important that the incubation times for the images are similar. The intensity differs even within a single sample and the concentration of salt seems higher in gluten rich areas even if the salt has been distributed homogeneously during mixing of the dough. When considering the intensity of an image, the mean intensity of the whole frame has been used. This might not be the best approach since the image gets darker in some parts. For the time dependence test in fig 16 the intensity was followed in a specific gluten rich area and starch area, data in fig A2-A3 in appendix. The problem with this approach was that the studied regions changed over time and made it hard to get similar areas for all images.

Another aspect that made the testing hard was that the sample moved over time. This resulted in a constant adjustment in the z-direction to keep focus in the same position of the sample. The fact that the probe was dropped on the sample, added an uncertainty of the specific concentration of the sample surface. For the tests where the probe was diluted in DMSO the surface was dried with paper before analyzing. In worst case the intensity can differ within the sample due to different probe concentration rather than salt concentration, although the probe seems to diffuse into the sample. The probe is binding specifically to the sodium ions, which means that the salt has to be dissolved to be detected. This was not a problem in the homogeneous systems due to the high water content, but the dough contains less water which could decrease the dissolution of salt particles. For all dough salt with small particle size has been used which would make them easier to dissolve.

Study dough is hard, and the system differs from the homogeneous systems. Further research has to be done to enable reliable results for determination of salt content and inhomogeneous distribution. The following proposed method is a starting point for further evaluation, but needs further development.

- Prepare the dough an place a small piece from the dough center in a metal cup. Add one drop 30μM probe diluted in ethanol to the dough surface and leave to dry.
- Let the dough with probe solution incubate for at least 30 min before adding a cover glass and start analysis. Use 10x objective with zoom 1-4 depending on the studied magnification.
- Use the following settings: format 1024x1024 to get better resolution, line average 8, AOTF 25%, adjust gain to get a balanced image.
- Both time dependence and affinity have to be taken into account. Always take images over time to capture changes in the intensity. Keep in mind that the affinity to starch and gluten differs, always compare to images with background fluorescence.
- Only compare images with the same setting and the same incubation time.

4.2.5 Creation of 3D-projections for bread studies

For the studies in bread focus was to find settings and a method to visualize the structure and how different types of projections can be used to study the distribution of salt. To find a good method for creating a 3D-projection of the studied bread sample three different aspects was considered. The total depth in the sample, distance in z-direction between each layer in the stack and different line average. Two different objectives was used for the bread studies, 5x and 10x. To create a 3D-projection a stack of images was taken at the same position but at different depth. The images in the stack were used to create a maximum projection that shows the maximum intensity for each pixel throughout the whole stack. This gives a projection with a 3D character.

To compare projections from different samples they have to be created in the same way. The effect of the distance in z-direction between each image in the stack was evaluated in a span from 30-140μm. The distance could be up to 75μm without any visible effects in the projection. The risk with too high gap between the images is to lose structure between the layers. Line average for the previous studied systems had been set to 8 both for homogeneous samples and dough. When a 3D-projection is created the images in the stack are put together and the pixels in the final projection comes from different images in the stack. This reduces part of the effect of scanning each row several times. The line average can be reduced to about 1-2 without effecting the final result. Projections with different total depth may highlight different parts of the structure and projections with smaller depth could be easier to study depending on the focus for the study. When a smaller depth is used the distribution in a specific layer of the sample can be studied without distraction from areas further down. When a total overview of the structure is interesting the whole distance that can be observed should be covered. Examples of projections with both different distance between the images in the stack, different line average and different total depth is presented in fig A9-A11 in appendix.

Study the distribution in a bread compared to a dough can be done by use of different magnification and several images of the same magnification can be put together to cover a bigger area. Different magnification has been tested in fig 24 from 5x in the left image to 40x in the right. Comparing the extreme images gives an idea of the different focus that can be used for studying bread. With a high magnification it is possible to see the starch granules and to analyze if there are any differences in the distribution between different components of the bread. Images with high magnification makes it possible to study salt distribution in a small area and to analyze the structure close up when inhomogeneous distribution is used. This can be helpful to find differences in the structure depending on the salt concentration.

With a low magnification as in the left image in fig 24 are the granules not detectable but it is possible to study if the distribution differs between different parts of the sample. To study an even wider area several projections can be put together like in fig 25. In this way can the inhomogeneous distribution be verified as previously shown by Noort et al [11] and Konitzer et al [12]. Both has used projections of a wide area to compare different salt distribution methods to verify that the concentration of salt differs in different positions depending on if small, big or encapsulated salt particles has been used.

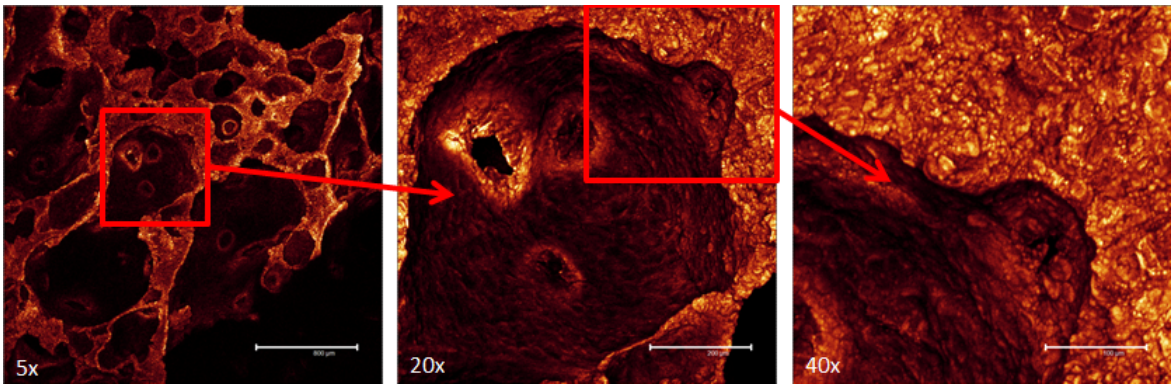


Figure 24: Projections of the same position in a bread sample with different magnification. The projections have 5x, 20x and 40x magnification and images was taken on Leica SP2 with AOTF 9% and gain 537. The white lines represents 800, 200 and 100 μm.

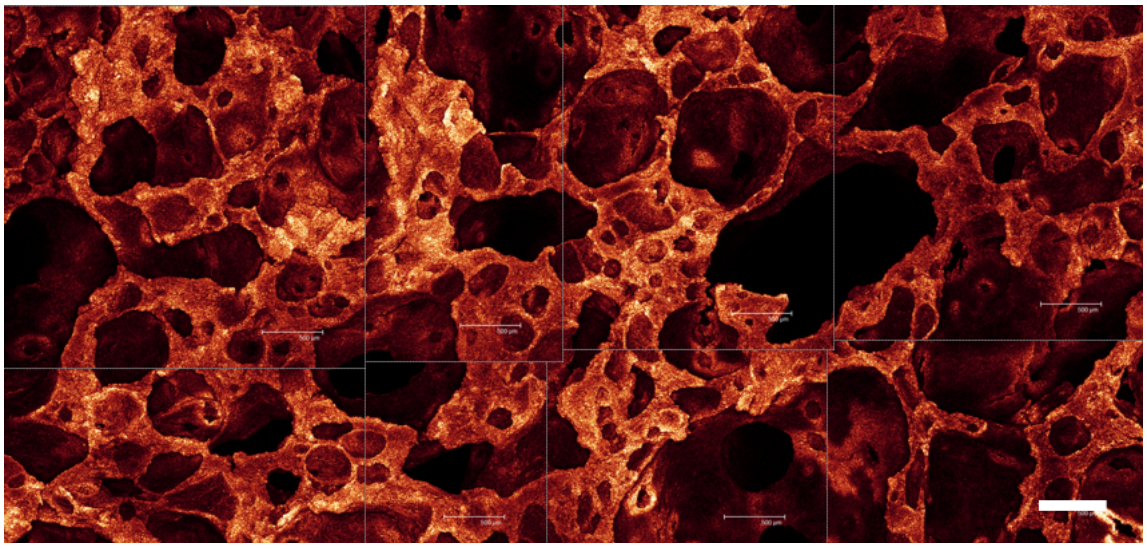


Figure 25: Several images put together to get an overview of a bigger area. The sample contains 2% salt and projections was taken at leica SP2 with AOTF 9% and gain 550. The white line in the bottom right corner represents 500 μm.

4.2.6 Factors that affect the 3D-projections, reduction of background fluorescence and study of bread with different salt particle size

Factors that affects the 3D projection and the intensity is the time dependence, the diffusion of the probe and the background fluorescence. The time dependence and background fluorescence has been studied for the previous model systems and had to be investigated in bread to find out the extent of their influence. Diffusion was investigated to find out if the probe diffuses in the sample or stays at the location where it has been applied. If it does not diffuse in the sample the amount of applied probe in a specific spot might influence the result and give a false indication of high salt concentration. The studies in salt solutions in fig 9 shows that a higher probe concentration most likely give a higher fluorescent signal. This means that if the probe distribution is uneven, differences in the intensity might come from changes

in the probe concentration rather than salt concentration.

The time dependence has been investigated in a similar ways as the other model systems, but it was studied in a full 3D projection instead of a single layer. The result is presented in fig 26 with projections from 3 to 48 min after the application of the probe to the bread. The result shows an increase in the fluorescent signal up to around 30-40 min after the application of the probe. The fluorescence then stays stable for at least a few hours, that gives long time to analyze the sample without risk for changes in the response signal. The change in intensity can be connected to the drying of the bread when the sample in the first few frames is still fresh and soft but dries over time to become hard and fragile. Even if the softness of the bread changes, the structure is stable which means that a dried bread is comparable to a soft bread. Regarding salt content and distribution, one thing that has to be accounted for is that the edges of the bread rise if the sample is too long and not wide enough. This will create a surface that is not flat and affects the distance between the objective and the sample.

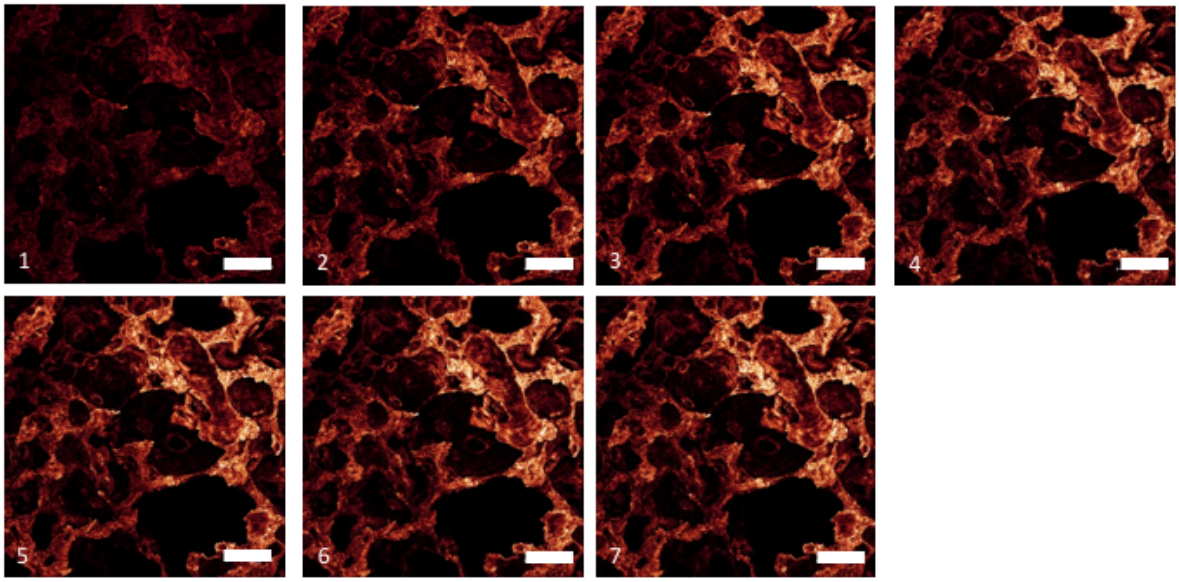


Figure 26: Intensity changes over time. Projections was taken from 7-30 min after the probe was applied on the sample. The study was made on Leica SP5 with AOTF 8% and gain 506. The white lines represents 500 μm.

A test for diffusion of the probe was conducted with images taken at the edge of the area that the probe had been dropped on. 3D projections were taken from 7-30 min after that the probe was applied to the sample. The result showed no diffusion and the projections can be found in fig A12 in appendix. This indicates that there could be some changes in the intensity due to concentration differences of the probe concentration. The ethanol evaporates fast which could reduce the diffusion. If the probe has time to diffuse, it probably happen faster than the time it takes to find the edge and make the first projection. An alternative could be to delude the probe in DMSO as in Noort et al [11]. The risk is that the application of the probe could affect the bread structure since DMSO does not evaporate and might get the bread soggy.

Background fluorescence of the probe in a bread without salt was investigated and compared to bread with 0.1 and 2% salt. All bread was baked with tap water and ordinary table salt

and the 3D projections can be found in fig 27 and fig A13-A14 in appendix with 0% to the left and 2% salt to the right. The projection with 0% salt shows clear structures even if the intensity is lower than for both breads with salt. This intensity has to be removed to give a correct image of the salt distribution and salt content if the method should be used to determine the concentration of the salt.

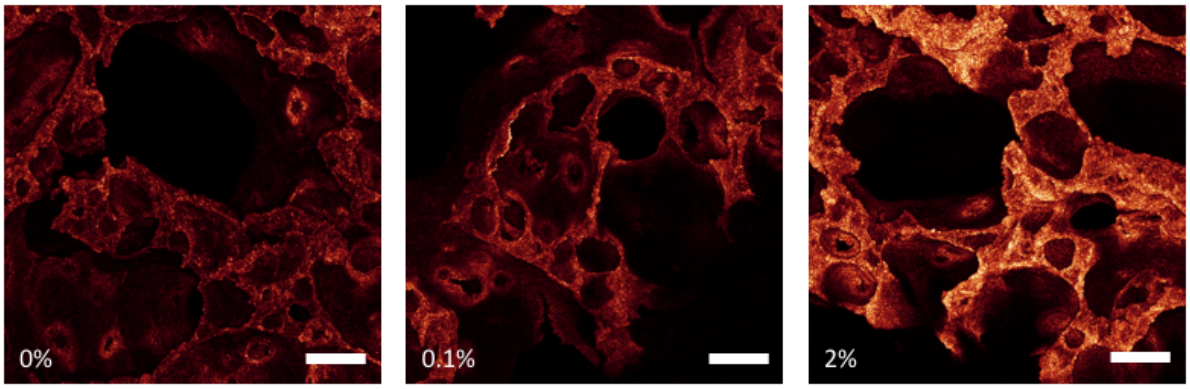


Figure 27: Bread with 0, 0.1 and 2% salt. Studied with Leica SP2 with AOTF 9% and gain 576.8. The white lines represent 500 μm.

A program was created to remove the background intensity from the sample with 0% salt from the ones with salt. Each projection contains a stack of images in different z-levels. The program considered each image separately and the intensity was set to 25 to mark the limit for where there where structure in the reference sample with 0% salt. Each pixel in every z-level image with a value higher then 25 was considered as structure and a mean value for those pixels represents the background intensity. A binary image of a mean projection of the stack with 2% salt where white pixels is structure with higher intensity than the mean from the reference sample was created. The binary image was multiplied with the original mean projection to get a final image without the background fluorescence. The original image, the binary image and the final mean projection for a bread with 2% salt can be seen in fig 28. The difference between the original projection and the final projection is noticeable, but the main structures are still the same. To further evaluate the image a histogram was created for the final projection, data not shown. The histogram shows the intensity distribution within the projection and can be used to compare projections with different salt concentration. Later on, projections with different salt distribution can be studied to find out if the distribution method works. Histograms from samples with homogeneous distribution of salt can be compared to histograms with uneven distribution to evaluate the distribution methods. It can also be used to find out what concentration of salt there is in different positions of the sample by comparing the number of pixels with different intensity.

So far bread baked with salt of small particle size has been used. Konitzer et. al [12] used uncoated salt particles on bread baked with larger salt particles according to table 9 and was compared to bread with salt of small particle size. Projections was created over a large area like in fig 25 to be able to capture the inhomogeneous distribution. The images can be found in fig A15-A15 in appendix where the image with large particles is brighter the the one with small particles. The same settings was used and both samples was studied after more than 40 minutes which, according to previous tests, would result in stable intensity. It is possible that

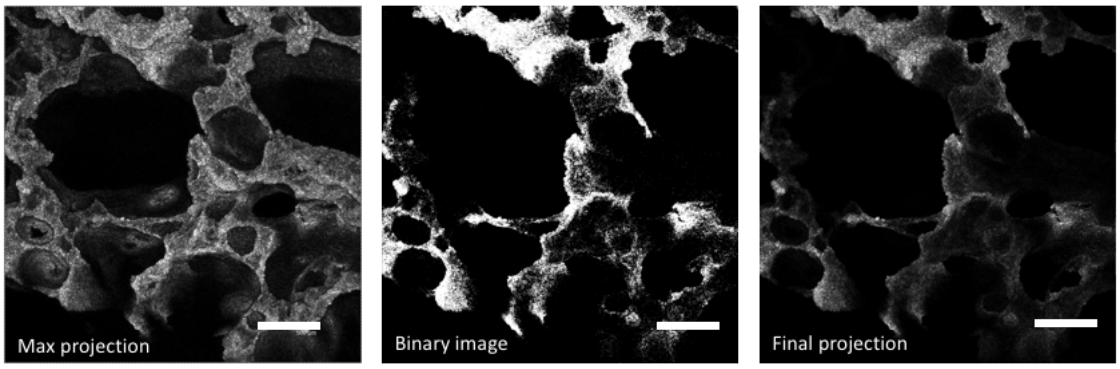


Figure 28: The original projection, the binary image and final projection after reducing the background fluorescence. The sample contains 2% salt and was studied on the Leica SP2 with AOTF 9% and gain 576.8. The white lines represents 500 μm.

the difference in intensity between the two samples comes from a difference in concentration at the studied position due to the two particle sizes. Unfortunately does the sample with big salt particle appear to have homogeneous salt distribution. The bread studied in Konitzer et. al [12] used colored salt particles which made it possible to find a location with both high and low salt concentration. The salt in this study was added in the beginning of the mixing which could contribute to a solubility of the salt during the mixing, resulting in homogeneous distribution in the bread.

4.2.7 Important factors for bread studies and proposed method for further studies

For studies in bread it is important to make sure that the settings are adapted to the specific propose of the image. The settings should be kept constant for the projections that is compared to each other. Due to the different levels and the porous structure a good approach is to get an overview of the sample by making several projections in a row to cover a wide area. This is important when inhomogeneous salt distribution is studied and a big area has to be covered to capture the variation in salt concentration. One problem with the method is that the probe does not diffuse into the sample. That is good in the way that the concentration at the studied position stays high so lower gain and laser can be used. It is bad in the way that the fluorescence intensity can differ depending on the probe concentration and provide a result that looks inhomogeneous although it's not. This can be seen in fig 25 where the right end side of the image is on the edge of where the probe has been applied and appears darker than the middle and left part of the image.

3D-projections is a good way to study the structure and salt distribution in bread. The bread studies were performed at two different microscopes, with small differences in the settings. An optimized method will be applicable for both objectives, but the method is mainly developed for Leica SP2.

- Cut out a sample from a bread slice with a sharp knife. Make sure that the sample has a flat surface and that it is not to thin so the edges rises during drying.

- Drop a few drops 30 μM probe solution diluted in ethanol on the bread, distribute the drops evenly on the sample surface. Let the bread dry for 30-40 min before analyze.
- Use either a 5x or 10x objective, don't use cover glass, the sample has an uneven surface and a dried sample will not attach to the cover glass. Use zoom 1-4 depending on the preferred magnification.
- If projections are made of a wider surface, move the objective in the same direction for a preferred number of projections. Then move the objective perpendicular and go back the same number of images. Make sure that there is an overlap between each projection to enable merging to visualize the bigger area.
- To create the 3D-projection: use 50-75μm between each image in the stack. Take images from the top of the sample and as far down as possible. Use line average 1-2 and when the stack is complete, create a 3D-maximum projection.
- The following settings are proposed: AOTF 7-10% and gain 500-600. Make sure that the same settings are used for all projections that are compared to each other.

5 Conclusion

The conclusions of this project are that the method seems to work. A wider knowledge about the probe CoroNa Green and its behavior in both homogeneous systems, dough and bread has been gained. The probe has, to our knowledge, not been used in studies of dough before and only once for bread studies. This work contribute to the understanding of the possibilities and limitations of an image based method for detection of salt content and distribution has for these systems. Different conclusions can be made from the solutions, gels, dough and bread.

For homogeneous systems it is possible to get a good overview that works well within the studied salt concentration interval. One important parameter to keep in mind is that the concentration of the probe influences the result. The concentration of the probe in the sample should be known and be the same for all compared samples. It is important to evaluate the time dependence if a semi-solid systems is studied with an inhomogeneous probe concentration.

Dough is a hard system to study due to influence of several parameters that contribute to uncertainties in the result. The most important factors are the diffusion of the probe that contributes to a time-dependence and the affinity to different components. The method is working, but it is very sensitive to changes and all parameters has to be the same to enable comparison between samples. The method worked for determination of inhomogeneous salt distribution by detecting differences salt concentration over a wider area, which is a good result. Another important aspect achieved from the dough studies is that the probe is sensitive to high temperatures and is not working over 65°C.

A 3D-projection method was successfully developed that can be used to study salt distribution in bread. Bread is less sensitive to changes compared to the dough and as long as the incubation time is long enough the system stays stable and provides reliable results. The background fluorescence has to be reduced to enable determination of the specific salt concentration.

5.1 Outlook/further research

For further projects the inhomogeneous salt distribution in both homogeneous and heterogeneous model systems has to be studied to find out e.g. if it is possible to track the dissolution of a salt particle. This can be useful to gain further understanding of the function of the probe in different conditions. Different types of salt distribution in bread with some of the methods that has been discussed earlier in this report. Both encapsulation of salt and addition of bigger salt particles in the end of the dough mixing is methods that would be interesting to get the result from. A good way to visualize where the salt is located is by coloring the salt in a similar way as in Noort et al [11, 13], or Konitzer et al [12]. In that way pieces of the bread can be cut out and studied where it is certain that the salt particles is located. To study the affinity of salt to amylose and amylopectin is another area that can be studied to gain deeper knowledge of the salts behavior in a dough and bread. Comparison with a study conducted with another technique like ESEM is a way to validate the method and find out if it is accurate.

For further support of the data from this project could longer time tests for the dough systems be studied to find out if the signal stabilizes. If dough is considered an interesting system to

study, it is also necessary to find a good way to reduce the background fluorescence. For the bread systems is a comparison between dilution of the probe in ethanol and DMSO interesting due to that DMSO was used in the study from Noort et al. It would be nice to find out if the choice of dilution agent has an influence on both the structure of the bread and on the time dependence to get a stable signal as well as the diffusion.

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A Appendix, complementary images and graphs

A.1 Example images of solutions and gels

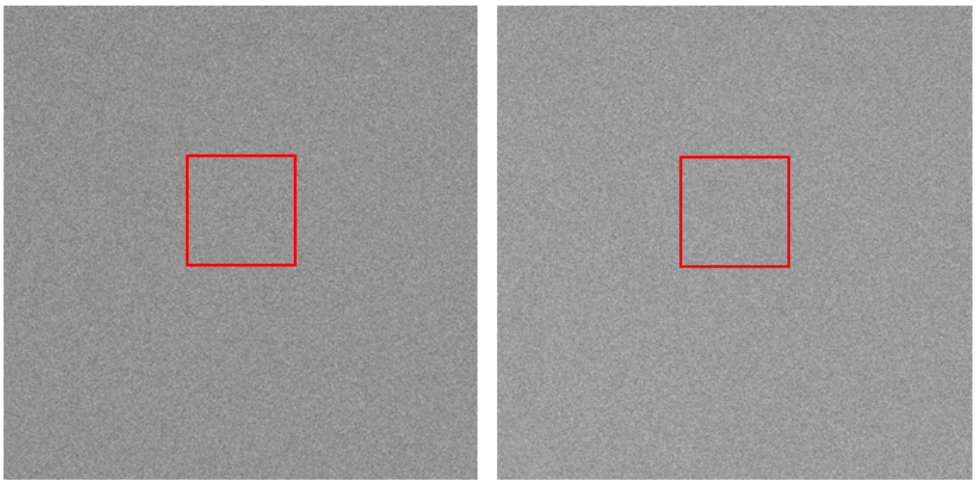


Figure A.1: Left image: Solution with 0.5% salt, AOTF 10%, gain 573. Intensity approximately 150 in the marked red area. Right image: Gel with 0.5% salt, AOTF 10%, gain 502. Intensity approximately 150 in the marked red area.

A.2 Time dependence in dough, complementary data for starch and gluten areas as well as confocal images

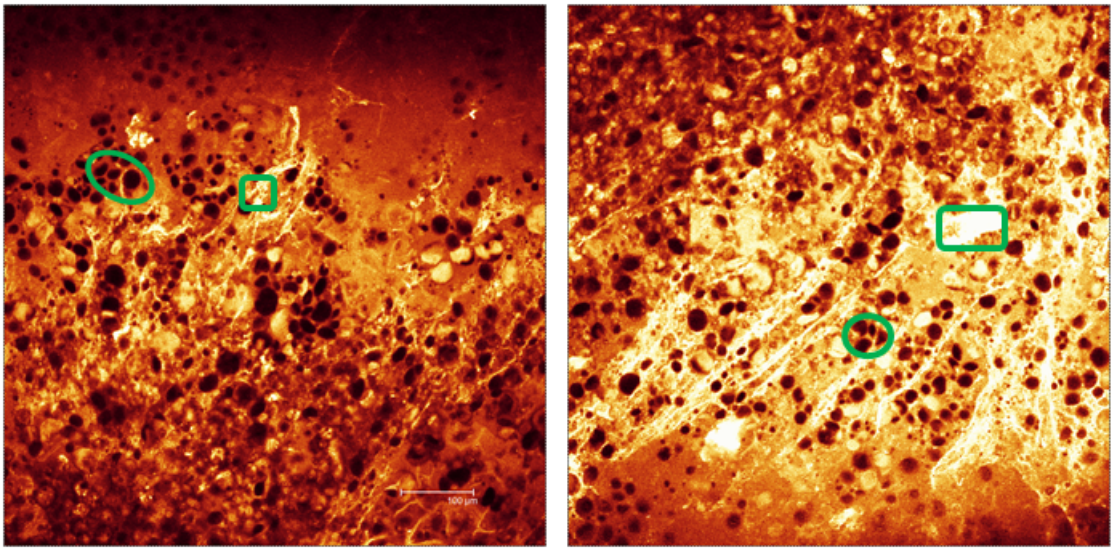


Figure A.2: Example of the studied gluten and starch areas in two of the time series. The gluten areas are marked with green squares and the starch are marked as circles. The intensity changes over times was studied for these specific regions.

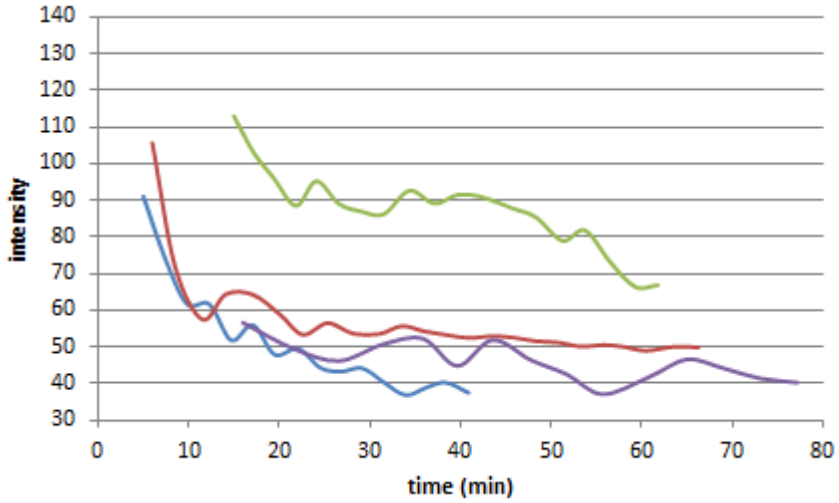


Figure A.3: Intensity changes over time where the intensity was studied in a few starch granules, as the regions shown in fig A.2

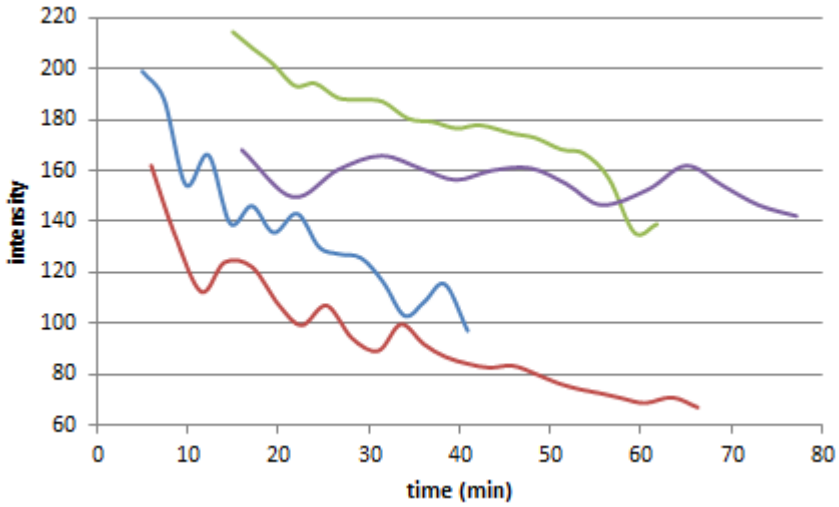


Figure A.4: Intensity changes over time where the intensity is measured in gluten rich area, as shown in fig A.2

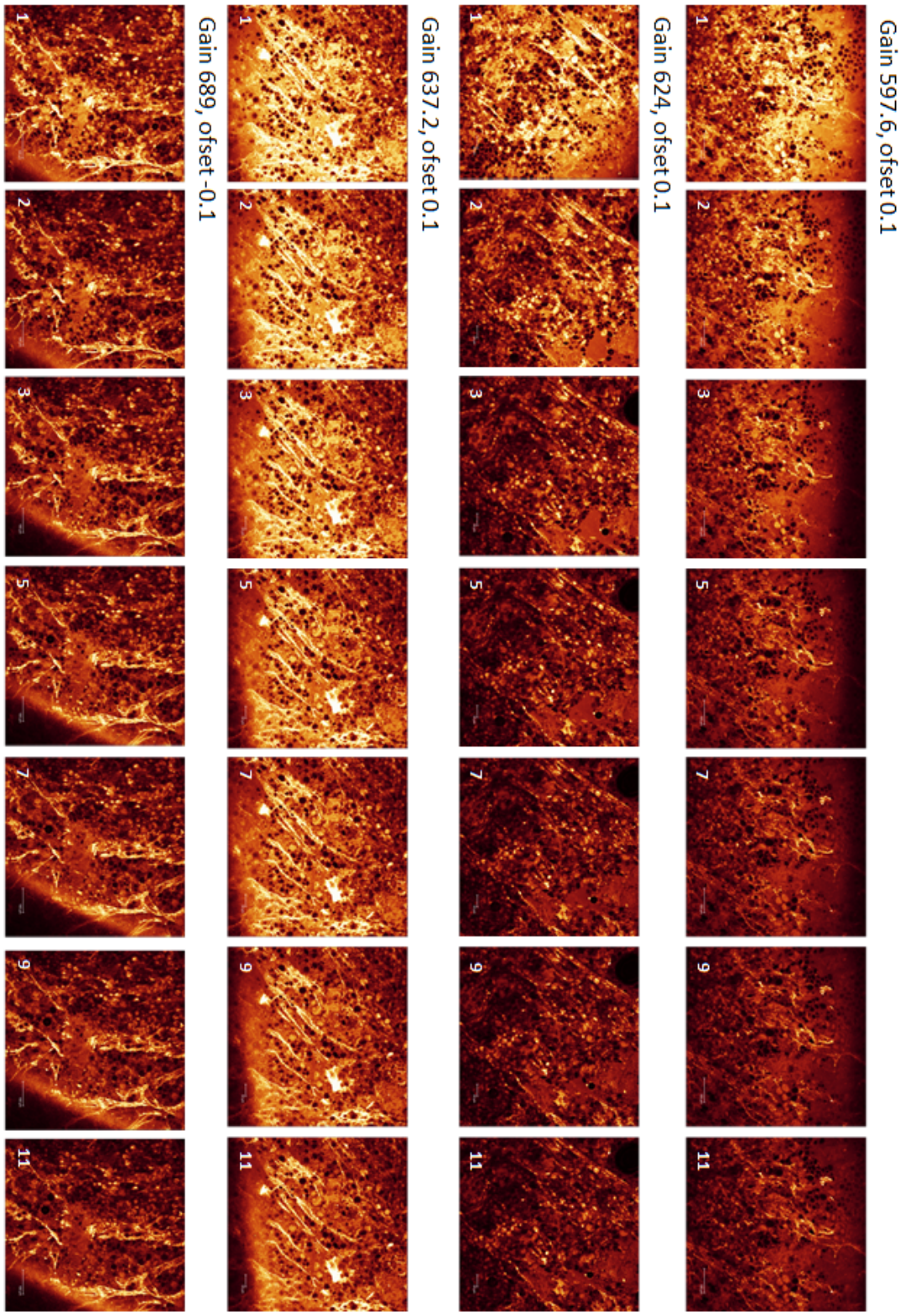


Figure A.5: Confocal images for the four time series. The gain and offset for each sample is stated above the image. Each row corresponds to one color in fig A3 and A4. Row 1: blue line, row 2: red line, row 3: green line, row 4: purple line. All images used AOTF 40% and the probe was diluted in DMSO and used in the conc 30 μM.

A.3 Different concentrations studied in dough with images taken over time, graphs for two replicates and confocal images.

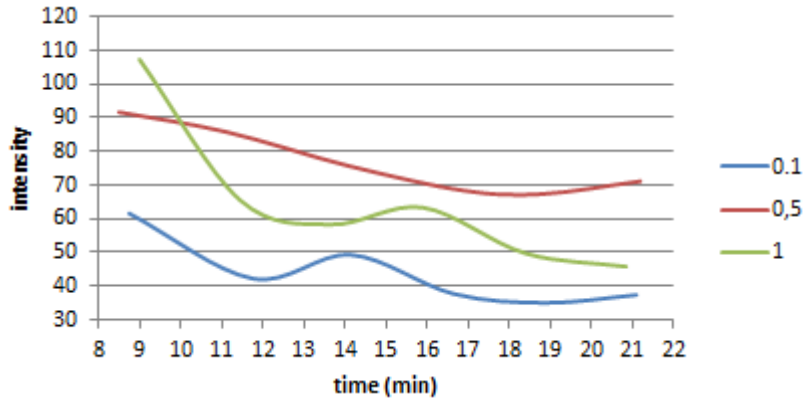


Figure A.6: First replicate for test with 0.1, 0.5 and 1% salt, gain 710, offset 0.4 and laser 30%, with settings according to tab 13. The graph shows the intensity over time from 9 to 21 min after that the probe has been applied to the sample.

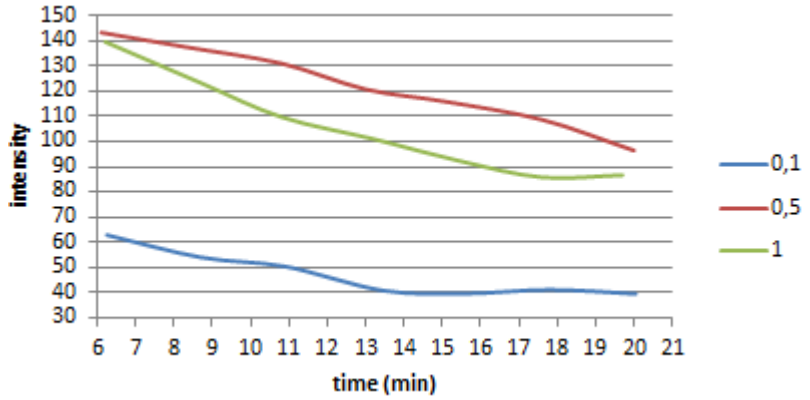


Figure A.7: Third replicate for test with 0.1, 0.5 and 1% salt, gain 672, offset 0.1 and laser 35%, with settings according to tab 13. The graph shows the intensity studied over time from 6 to 20 min after that the probe has been applied to the sample.

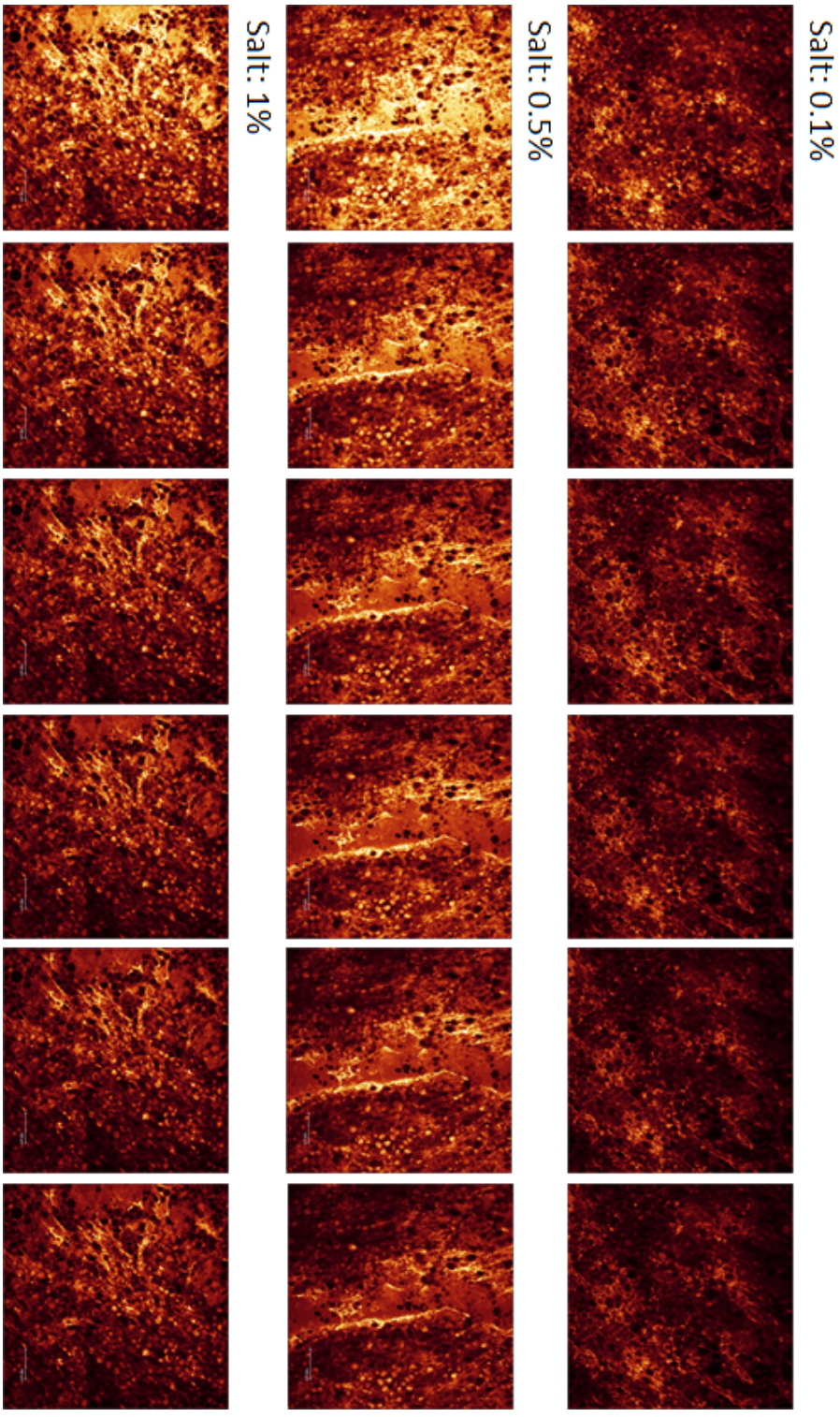


Figure A.8: Confocal images for replicate two of test with 0.1, 0.5 and 1% salt, gain 653.9, offset 0.1 (graph in result), with settings according to tab 13. Images taken from 3 to 18 min after that the probe has been applied to the sample.

A.4 Evaluated parameters for creating of 3D-projections in bread

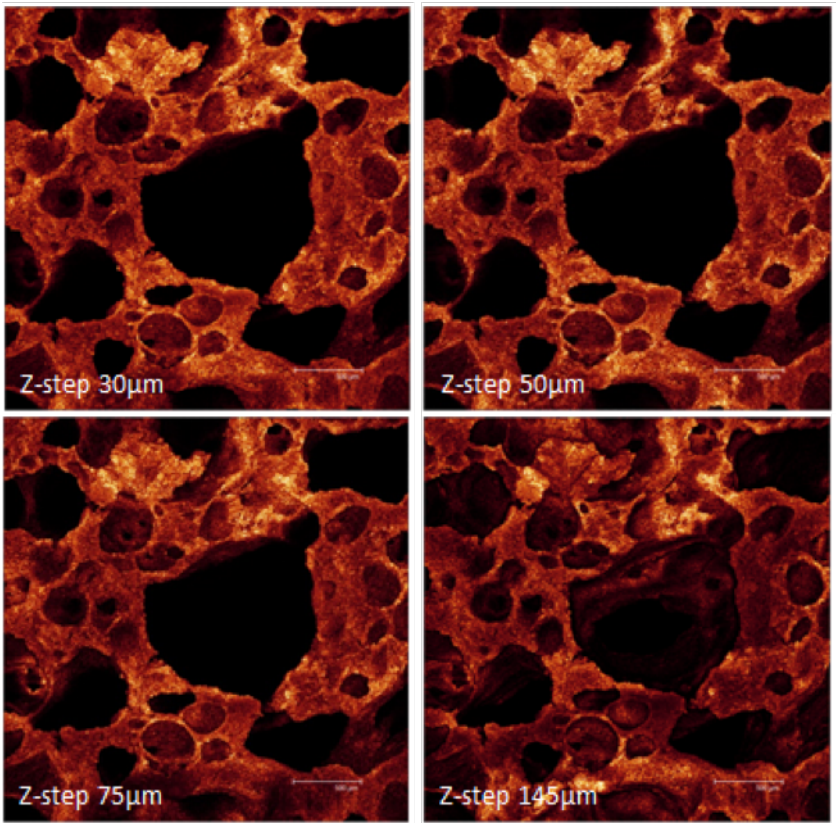


Figure A.9: Evaluation of different distances in z-range between each image in the stack used to create the 3D-projection. 30, 50, 75 and 145 μm was tested.

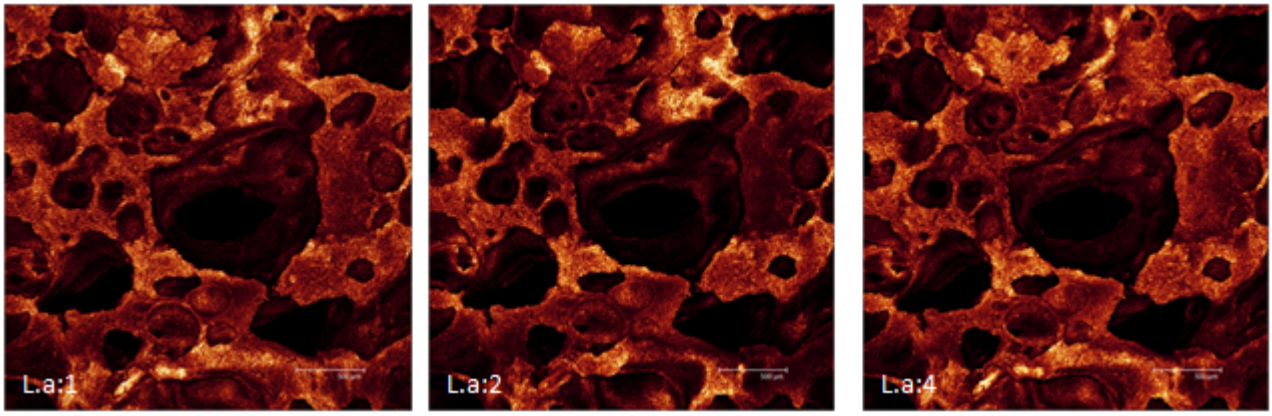
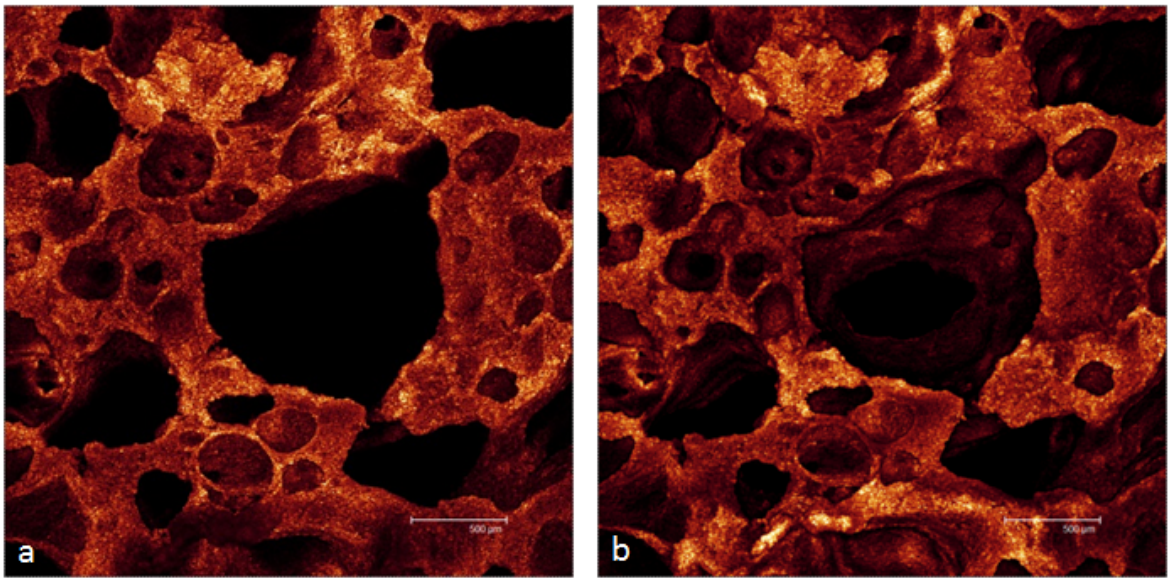
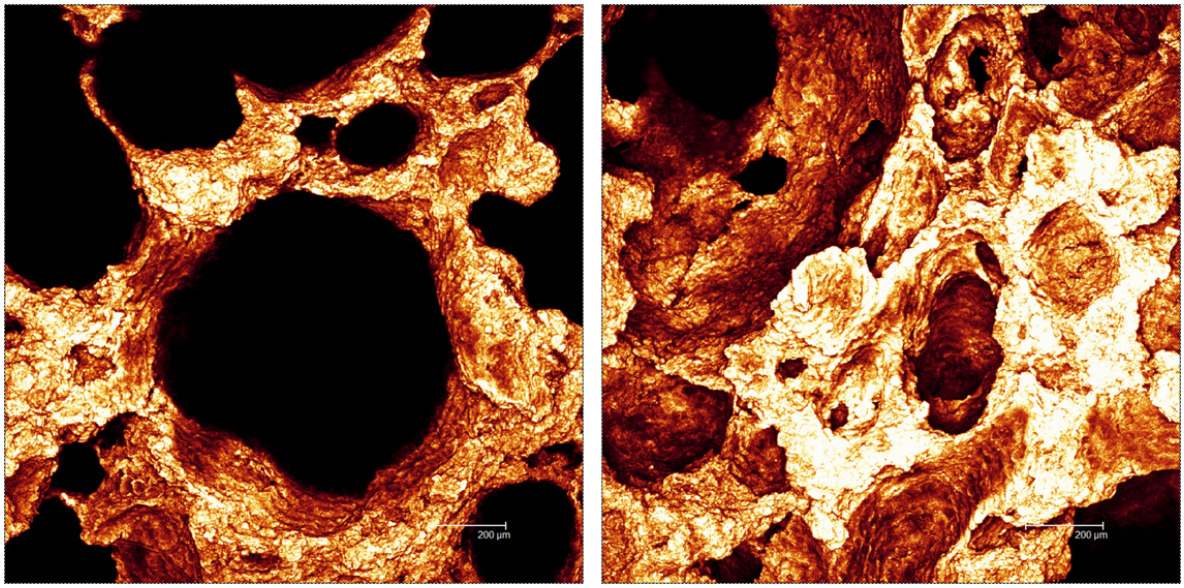


Figure A.10: Evaluation of the effect by use of different line average for the final 3D-projection. Line average 1,2 and 4 was evaluated.



((a)) Difference in 3D-projection from the same position with 400 vs 1000 μm in total depth.



((b)) Difference in 3D-projection from different positions with 600 vs 1200 μm in totla depth.

Figure A.11: The effect of using different total depth in the image.

A.5 Evaluation of diffusion in bread of the probe diluted in ethanol

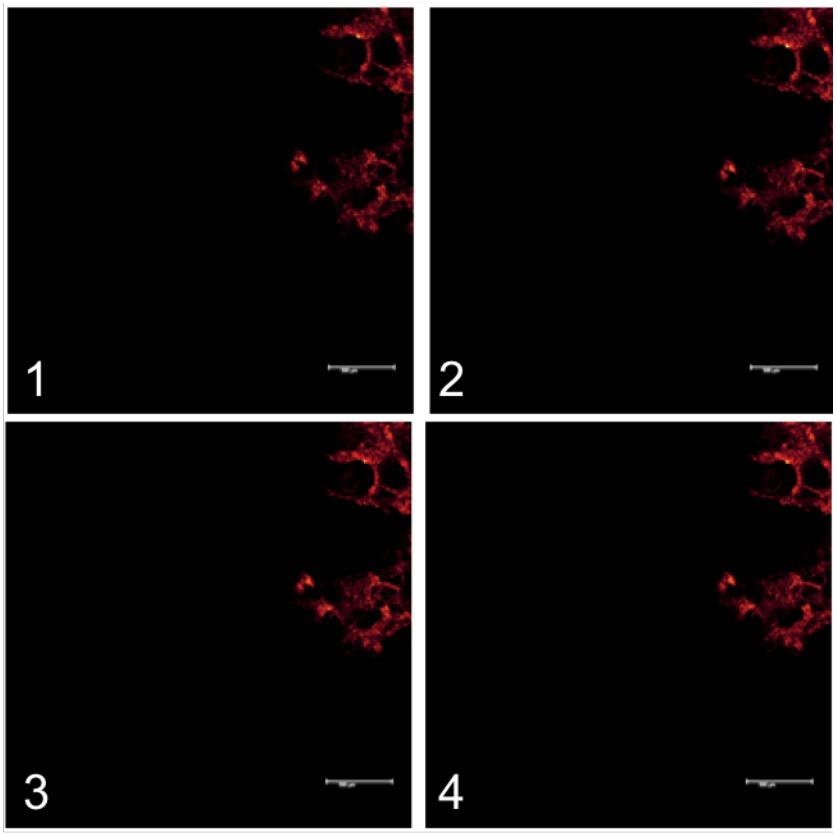


Figure A.12: 3D projections from test for diffusion of the probe. Projections created over time from 7-30 min after that the prove was applied to the sample surface. The projections are created at the edge of the are where the probe was applied. Structure of the bread is seen in the top right corner of each projection.

A.6 Complementary 3D-projections of bread with different salt concentration

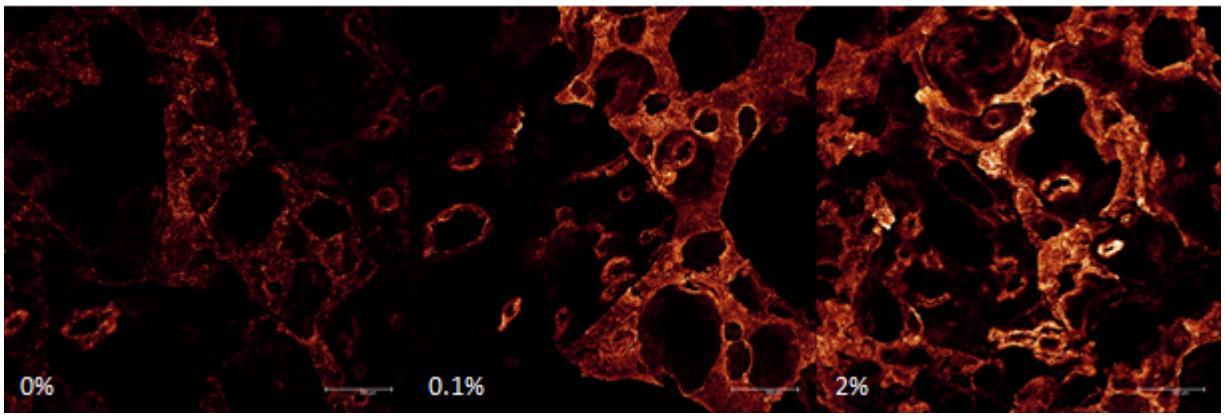


Figure A.13: Bread baked with 0, 0.1 and 2% salt. Studied with the Leica SP5 with settings according to tab 17. 3D-projections was created for a depth of around 1 mm.

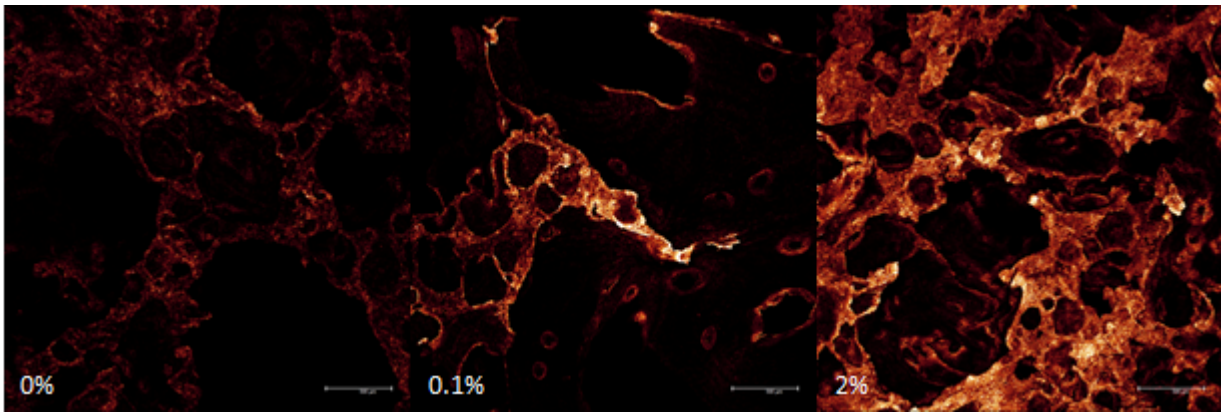


Figure A.14: Bread baked with 0, 0.1 and 2% salt. Studied with the Leica SP5 with settings according to tab 17. 3D-projections was created for a depth of around 1 mm.

A.7 Use of different particle size in bread with 2% salt

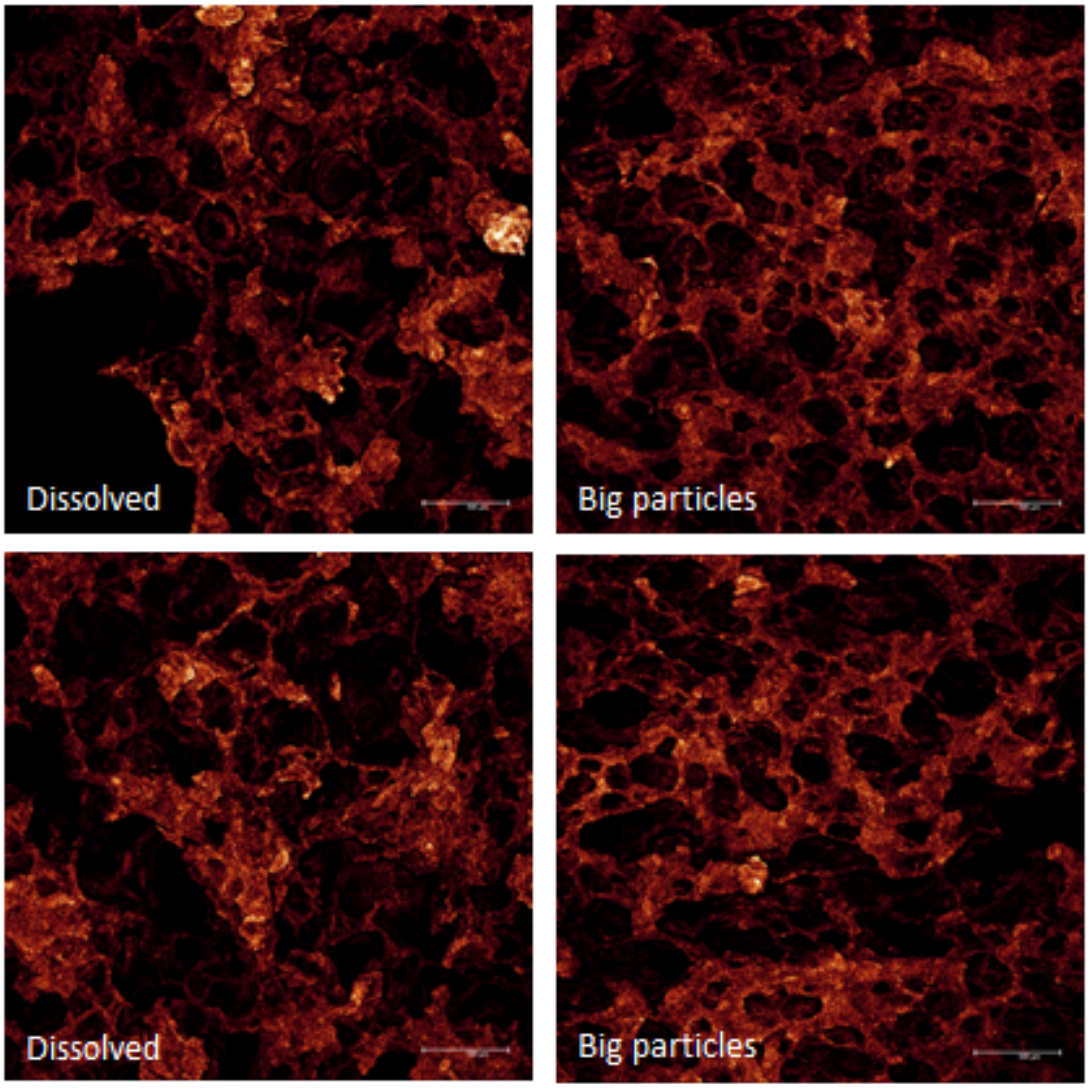
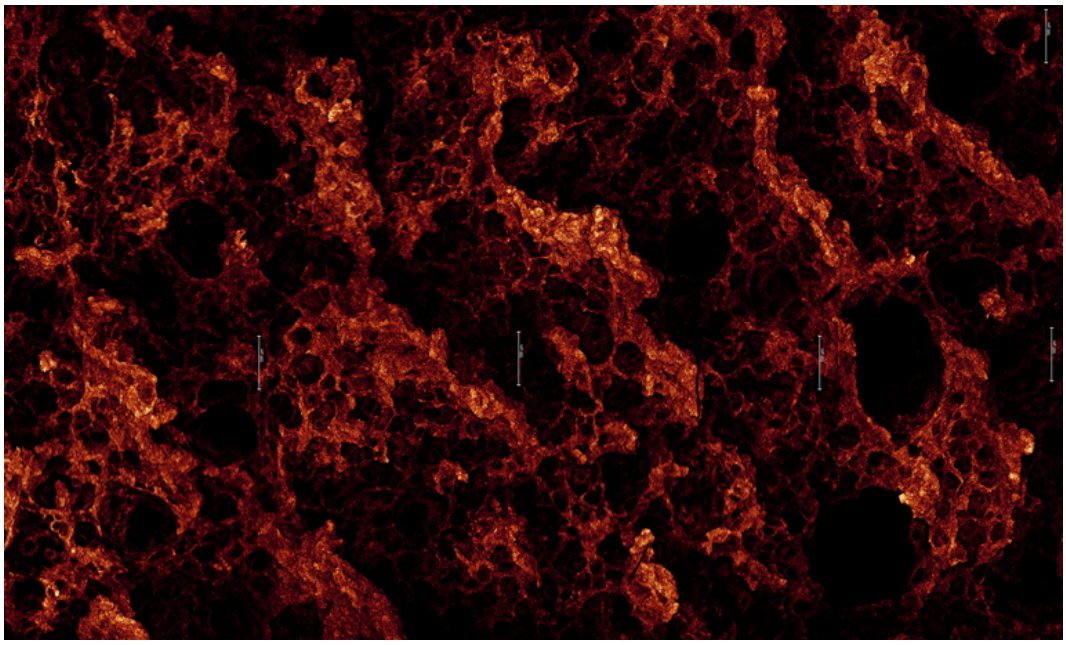
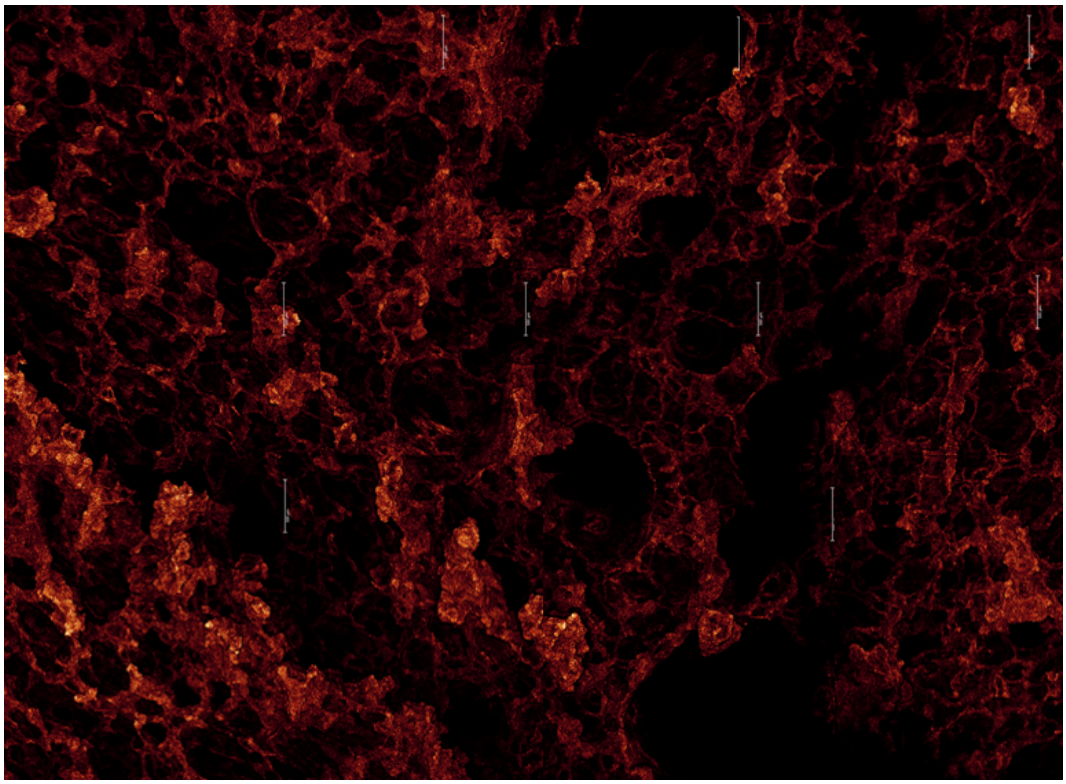


Figure A.15: Comparison of 3D-projections created in bread with 2% salt. The salt has either been dissolved in water before mixing with the flour or salt of big particle size, up to 1 mm in diameter added to the flour was used.



((a)) Bigger area of bread baked with large particle size, up to 1 mm in diameter. Bread was studied with the Leica SP5 and settings according to tab 17.



((b)) Bigger area of bread baked with small particle size, 0.1-0.3 mm in diameter. Bread was studied with the Leica SP5 and settings according to tab 17.

Figure A.16: Study of two different salt particles over a large are in bread containing 2% salt.