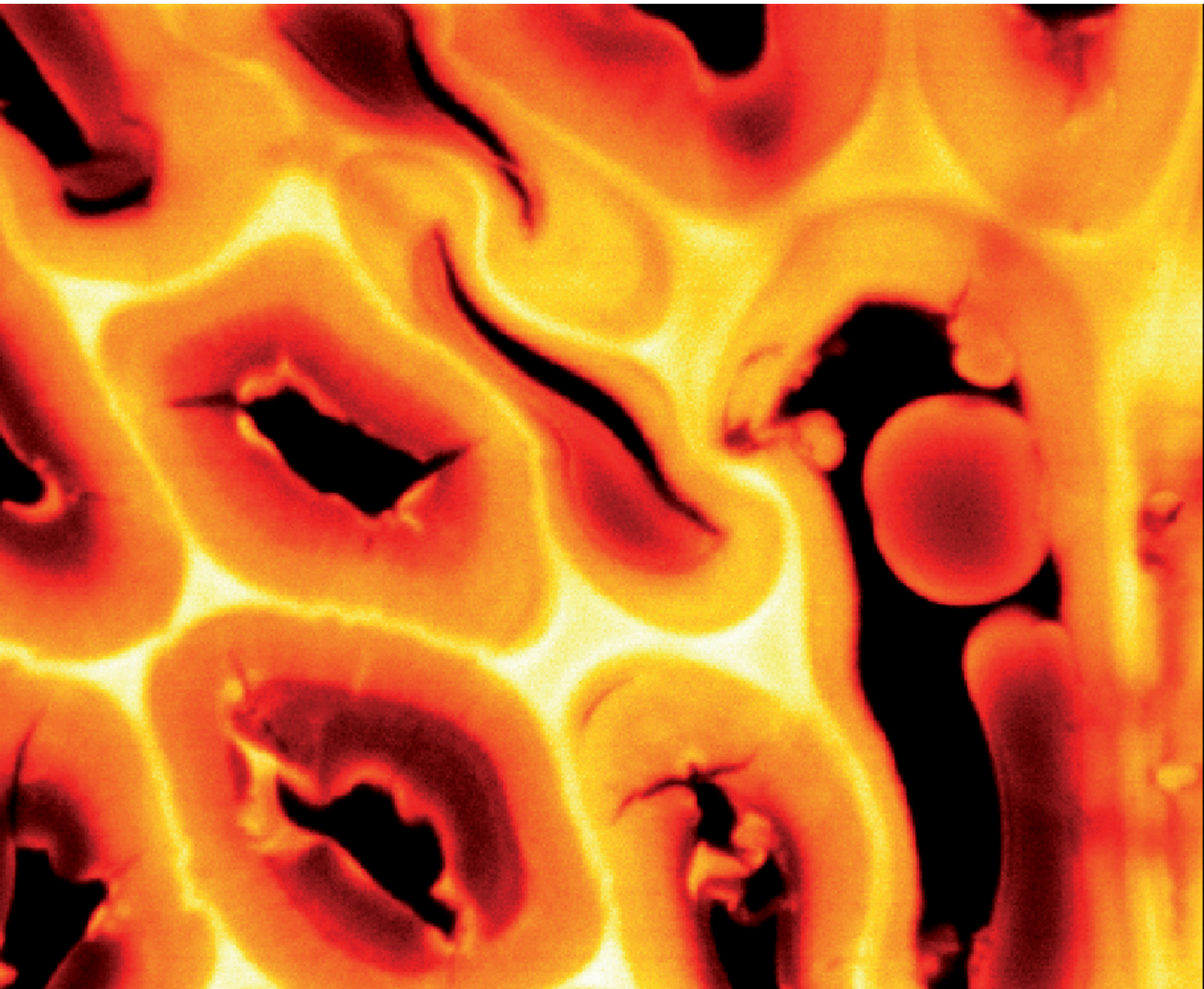
Oppdragsrapport 07/2010
fra Skog og landskap

RESEARCH REPORT ON CONFOCAL LASER SCANNING MICROSCOPY STUDIES ON FURFURYLATED WOOD SAMPLES

Andreas Treu

skog+ landskap
NORSK INSTITUTT FOR SKOG OG LANDSKAP



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Omslagsfoto: Fluorescence image of furfurylated beech, Fotograf: Andreas Treu, Skog og landskap

Norsk institutt for skog og landskap, Pb 115, NO-1431 Ås

SUMMARY

Furfurylation of wood samples can lead to different outcome (different chemistry) and product properties depending on parameters such as pH-value, catalyst, amount of water, time and temperature. Changes in the furfurylation process can even lead to a deterioration of product performance, such as lower durability in comparison to other furfurylated products.

How is it possible to detect these changes in chemistry of a furfurylated wood product?

To answer this question confocal laser scanning microscopy was used. The expectation was to detect different emissions of the auto fluorescent furfuryl alcohol if differences in chemistry exist. Additionally to the differences in emitted wavelength, the area in the wood structure of different emissions was under investigation. It was the aim to detect whether different product performance of a furfurylated product or different furfurylation processes could be related to changes in chemistry and this was expected to be visualized by CLSM.

SAMMENDRAG

Furfurylering av treprøver kan gi forskjellig resultat (forskjellig kjemi) og produktegenskaper avhengig av parametre som pH-verdi, katalysator, vannmengde, tid og temperatur. Forandringer i furfuryleringsprosessen kan til og med gi et dårligere produkt med mindre holdbarhet i forhold til andre furfurylerte produkter.

Hvordan er det mulig å oppdage disse forandringene i kjemien til et furfurylert treprodukt?

For å finne et svar på dette ble konfokal laserskanning-mikroskopi benyttet. Hvis det var forskjeller i kjemien, var det forventet å finne forskjellige emisjoner av autofluorescerende furfurylalkohol. I tillegg til forskjeller i avgitt bølgelengde ble området i treprøven som ga forskjellige emisjoner undersøkt. Målet var å finne ut om forskjellige egenskaper i et furfurylert produkt, eller forskjeller i fufuryleringsprosessen, kunne relateres til forandringer i kjemien. Dette var forventet å bli visualisert med CLSM.

Nøkkelord:	Konfokal laser scanning mikroskopi, furfurylering av tre
Key word:	Confocal laser scanning microscopy, furfurylation of wood
Andre aktuelle publikasjoner fra prosjekt:	-

CONTENT

1. INTRODUCTION

1.1. Statement of the problem

Furfurylation of wood samples can lead to different outcome (different chemistry) and product properties depending on parameters such as pH-value, catalyst, amount of water, time and temperature. Changes in the furfurylation process can even lead to a deterioration of product performance, such as lower durability in comparison to other furfurylated products.

How is it possible to detect these changes in chemistry of a furfurylated wood product?

To answer this question confocal laser scanning microscopy was used. The expectation was to detect different emissions of the auto fluorescent furfuryl alcohol if differences in chemistry exist. Additionally to the differences in emitted wavelength, the area in the wood structure of different emissions was under investigation. It was the aim to detect whether different product performance of a furfurylated product or different furfurylation processes could be related to changes in chemistry and this was expected to be visualized by CLSM.

1.2. Confocal laser scanning microscope (CLSM)

Confocal laser scanning microscope (CLSM) is a technique for obtaining high-resolution optical images. It has advantages over widefield optical light microscopy, including the ability to eliminate or reduce background information away from the focal plane, the ability to produce in-focus images of thick specimens, which means optical sectioning, in addition to the ability to control depth of field. One may consider the confocal microscope as a bridge between the conventional widefield microscope and the transmission electron microscope, regarding the degree of resolution. The resolution is somewhat enhanced over conventional widefield microscope, but considerably less than that of the transmission electron microscope. The enhanced resolution in confocal microscopy is due to the ability of excluding secondary fluorescence in areas removed from the focal plane from resulting images.

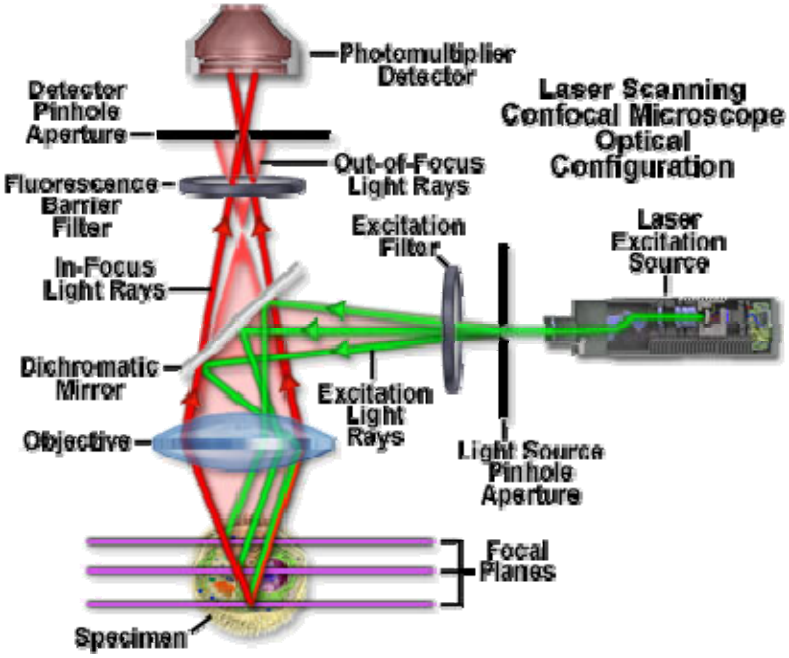


Fig 1 Schematic illustration of confocal microscopy

In a confocal laser scanning microscope, the laser beam diameter is limited by a pinhole aperture that is situated in a conjugate plane (confocal) with a scanning point on the specimen. As shown in Fig. 1 the laser beam is reflected by a dichromatic mirror (a beam splitter) and is focused into a small focal volume within a fluorescent specimen. Secondary fluorescence emitted from the specimen pass back through the dichromatic mirror and is focused as a confocal point at the detector pinhole aperture. After passing the pinhole aperture, the fluorescent light is detected by a photomultiplier tube, PMT. Out-of-focus light is blocked by the pinhole, making the resolution in confocal microscope increased. The light source is scanned over the specimen and the PMT transforms the light signal into an electrical one that is recorded by the computer for processing and display of images. The image has to be rebuilt from the point intensities according to the xy-coordinates.

The pinhole aperture mentioned above is one of the most important components in the confocal microscope. It acts like a filter in front of the photomultiplier allowing only light from the focal point to be detected. Because of this one may obtain images of various z axis planes of the sample, also known as z stacks. The pinhole aperture makes the specimen illumination different in the confocal microscope compared to traditional widefield epi-fluorescence microscope as shown in Fig 1.

The specimen is scanned with a finely focused spot that is centered in the focal plane. This makes high z-resolution possible.

2. SAMPLE PREPARATION:

Sample preparation has been an important factor during this study. Due to the hardness of the furfurylated material a microtome cutting of non-embedded material could not been used. The aim was to reduce the differences in surface topography of cross sections of wood samples to a minimum. Confocal Laser Scanning Microscopy doesn't need a thin cut section; instead wood blocs with dimensions of approximately 5 x 5 x 3 mm^3 were used. Only cross section surfaces were prepared and studied.

The furfurylated wood samples were cut in Cryotome at -20° C in order to get an even surface. It was not possible to get good results (good pictures) in the CLSM without preparing the surface. Even with cryotome the samples were difficult to prepare. A Teflon coated knife gave better results in preparation of the samples using the cryotome.

Since the sample preparation was improved constantly during the study, it can not be excluded that the improvement of sample preparation had an influence on the results.

Embedding of samples was also tested. The hardness of the resin (LR white) used for embedding was low compared to the hardness of the wood samples. A harder resin could probably improve the outcome. However, the cross section surface of embedded samples could be cut evenly with a diamond knife. Embedding gave therefore best surface properties but was only used for a limited amount of samples.

The majority of the samples was prepared by using cryotome.

3. SETTING OF EXCITATION AND EMISSION

Two lasers were used for excitation, Argon 488 nm and HeNe 633 nm. The detector ranges were 500-550 nm, 550-600 nm, 650-700 nm, 700-750 nm according to Thygesen et al. 2009.

The detectors were set in sequenced mode, which means that emissions from Argon 488 nm excitation were only detected in the emission range of 500-550 nm and 550-600 nm and emissions from HeNe 633 nm excitation were only detected in the emission range of 650-700 nm, 700-750 nm. Side effect can hereby be avoided.

The excitation gain was 590 V for Argon 488 nm and 772 V for HeNe 633 nm and was not changed during the test in order to compare better the emission pictures.

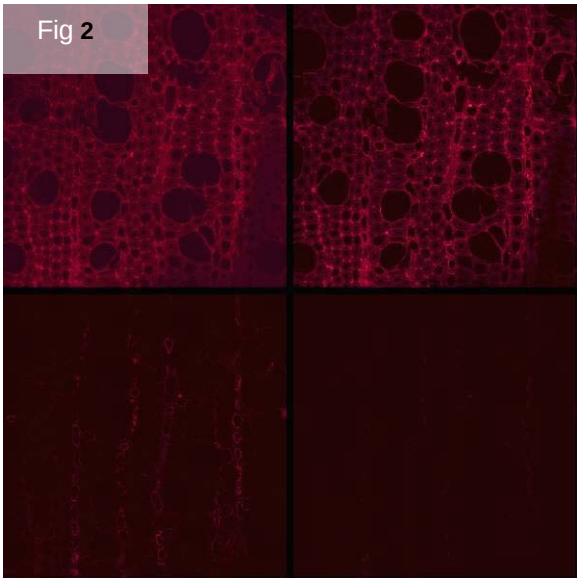
Images were recorded as averages of 16 frames in order to reduce noise.

The images can be interpreted due to color codes: color is going from black to red, to yellow and white with increasing emission intensity. Blue color indicates detector overload. The images can also be shown with 4 different colors representing the 4 different emission areas (see Fig **3**).

Table 1 Setting of excitation, emission and smart gain that was used in the beginning of the study for samples from marine testing

EXCITATION: ARGON 488 NM EMISSION DETECTOR RANGE: 500-550 NM SMART GAIN: 590 V	EXCITATION: ARGON 488 NM EMISSION DETECTOR RANGE: 550-600 NM SMART GAIN: 590 V
EXCITATION: HeNe 633 nm Emission detector range: 650-700 nm Smart gain: 590 V	EXCITATION: HeNe 633 nm Emission detector range: 700-750 nm Smart gain: 590 V

The initial pictures were taken from untreated beech samples in order to illustrate the auto fluorescent properties of lignin (see Fig **2**). The images of untreated beech wood samples (Fig **2** and Fig **3**) were taken with much higher smart gain (~ 1000 V). This was done in order get more information out of the sample. Without increasing the smart gain the emission of auto fluorescent lignin was hardly visible. It was therefore concluded that lignin is not disturbing the investigations on the auto fluorescence of furfurylated wood since the “noise” of lignin was insignificant.



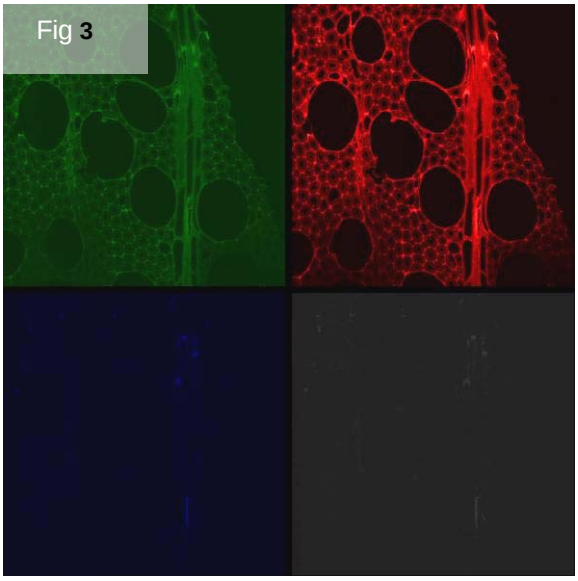


Fig 2 Fluorescence image of untreated beech wood (cross section, magnification 400 x)
Fig 3 Fluorescence image of untreated beech wood (cross section, magnification 400 x), using one color for each emission range

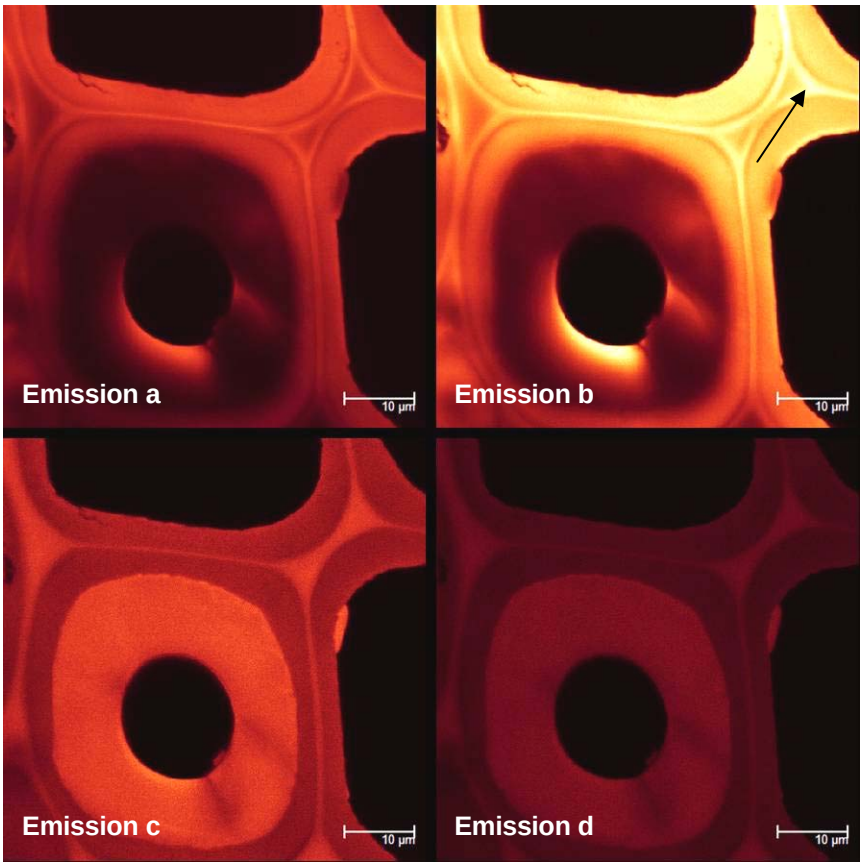


Fig 4 Fluorescence image of furfurylated Southern Yellow pine (FA 100, 630 x magnification oil objective, sample 194)

The 2 emission ranges (500-550 nm [a] and 550-600 nm [b]) from the Argon laser at 488 nm and the 2 emission ranges (650-700 nm [c] and 700-750 nm [d]) from the HeNe laser at 633 nm were used in the beginning of this study in order to reproduce the test setup of trials performed in earlier studies by Thygesen et al. 2009.

Emission a and emission d didn't give any additional information compared with the information obtained from emission b and c. Highest intensity of emission at 550-600 nm (b) can be seen in the middle lamella and the cell corners (see arrow). However, emission a couldn't give high intensities in all images that were taken later from other wood samples. Emission c shows also high intensity for the middle lamella, but not as distinctive as for emission b. Furthermore, the highest intensity in emission c images can be seen for fillings of cell lumina, whereas images of emission d don't give high intensities of signals at all.

This observation led to the reduction of emission ranges both in quantity as well as in range width. Only one emission range from one excitation laser was used for the last part of the study. 500-520 nm was used for Argon laser at 488 nm with a higher smart gain and 700-720 nm was used for HeNe laser at 633 nm.

In the following is the "green" color in images the emission between 500-520 nm excited by the Argon laser.

Bleaching, which can occur and will reduce then the emission of auto fluorescent material when excited for longer period, was not taken into account in this study.

4. CLSM ON FA-TREATED SAMPLES THAT FAILED IN MARINE TESTS

The furfurylated wood samples from marine water exposure in Sweden were rated with 4 (heavy attack by ship worm) and failed in test after one or two years (see table 2).

Table 2 Overview over furfurylated wood samples that failed in marine test

Sample	Wood species/treatment	Field test	Description
305	Beech FA 40	Failed in test	Rating 4 in 2009, 2 years exposure, severe attack by shipworm
380	Beech FA 70	Failed in test	Rating 4 in 2009, 1 year exposure, severe attack by shipworm
194	SYP FA 100	Failed in test	Rating 4 after 1 year, severe attack by shipworm

Fig 5 Furfurylated beech sample (FA 40, sample 305, rating 4 in 2009, 2 years exposure, severe attack by shipworm)

Statement of the problem: Why have these samples been rated badly after a short time of exposure (see Fig **5**) while other furfurylated samples from different processes have no or slight attack after many years?

Wood samples were cut out of the furfurylated samples that were exposed in sea water at the Swedish marine test station. Due to heavy attack of some wood samples it was not possible to cut out parts for CLSM always at the same position of a sample. Instead, samples had to be cut out where it was possible.

The investigation was started with untreated beech samples in order to illustrate the auto fluorescent properties of lignin (see Fig **2**).

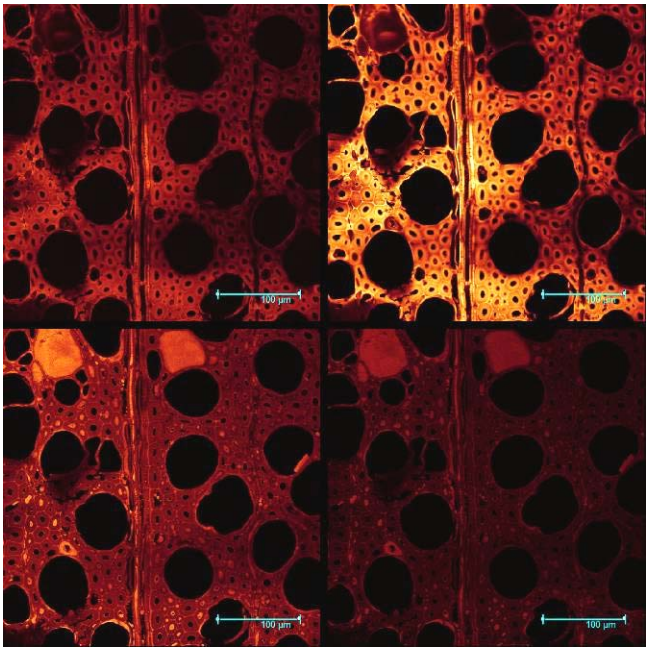


Fig 6 Fluorescence image of furfurylated beech (FA 40, 400 x magnification, sample 305)

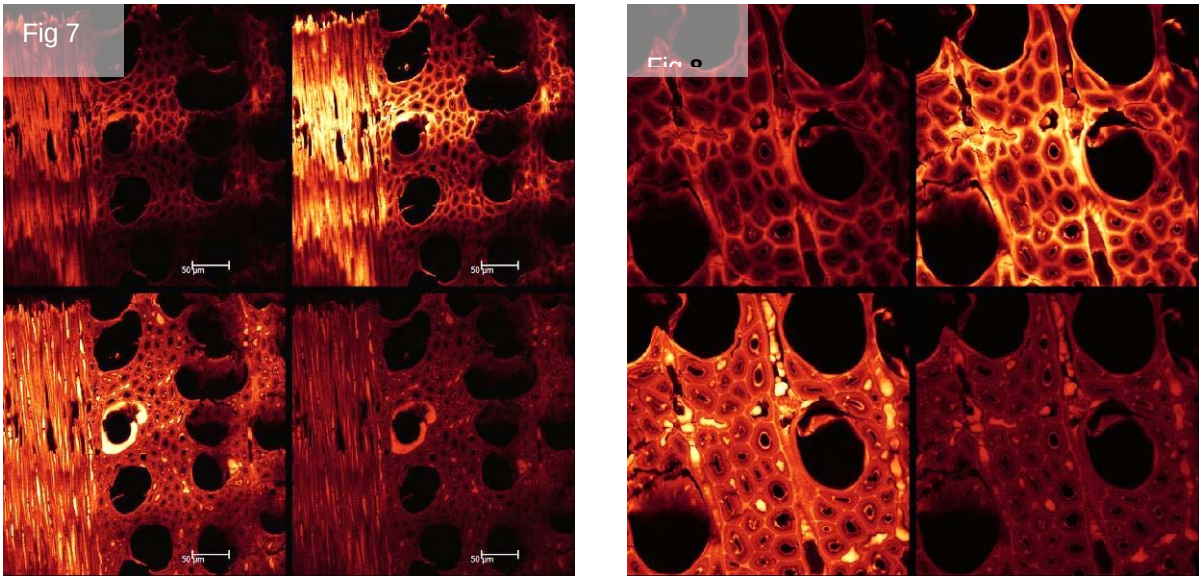


Fig 7 Fluorescence image of furfurylated beech (FA 70, 400 x magnification, sample 380)
Fig 8 Fluorescence image of furfurylated beech (FA 70, 400 x magnification, sample 380, zoom 2x)

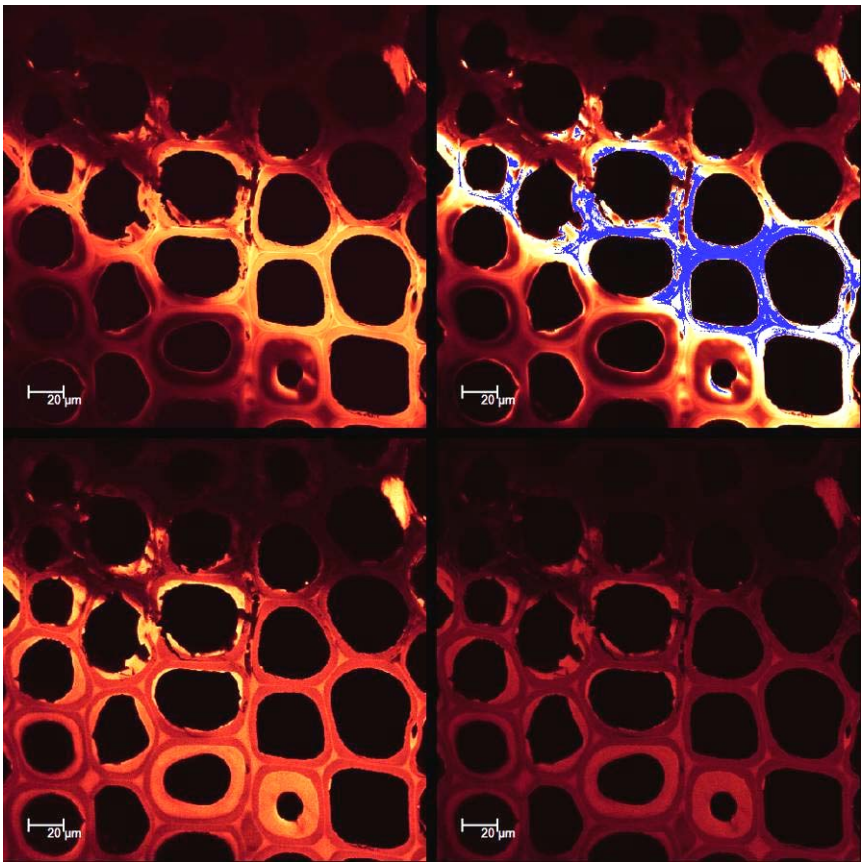


Fig 9 Fluorescence image of furfurylated Southern Yellow pine (FA 100, 630 x magnification oil objective, sample 194)

4.1. Preliminary results

- different concentration of FA could not be seen in the images as different emissions
- auto fluorescence of FA had its highest intensity in the middle lamella and cell corners
- different chemistry could be seen for two ranges of emissions
- High intensity of emission below 600 nm was indicating mainly middle lamella and cell corners
- High intensity of emission above 600 nm was indicating mainly fillings in cell lumina

5. CLSM ON FA-TREATED SISTER SAMPLES THAT DIDN'T FAIL IN MARINE TESTS

In order to compare the emissions of furfurylated samples reported in chapter 4 with furfurylated samples that performed well in a marine test, sister samples were used for this study. These sister samples were unexposed to sea water while the other pair of samples is still performing well in sea water exposure.

Table 3 Overview over furfurylated wood samples that were treated similar to sister samples that performed well in marine test

Sample	Wood species/treatment	Field test
B40-1	Beech FA 40	-
J2	Scots pine FA 70	-

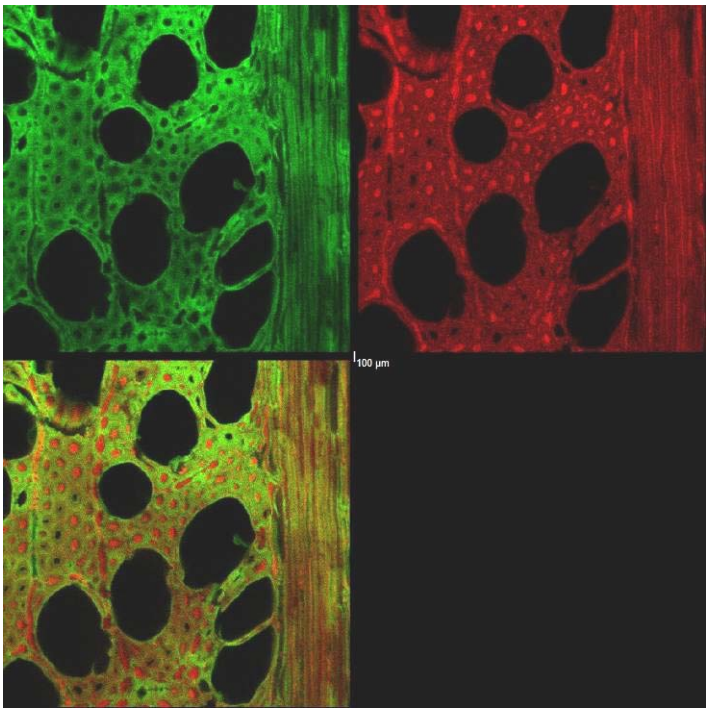


Fig 10 Fluorescence image of furfurylated beech (sample B40-1, FA 40, magnification 20x, zoom 2.87 x, emission 500-520 nm upper left and 690-720 nm upper right image, below image combining both emission ranges)

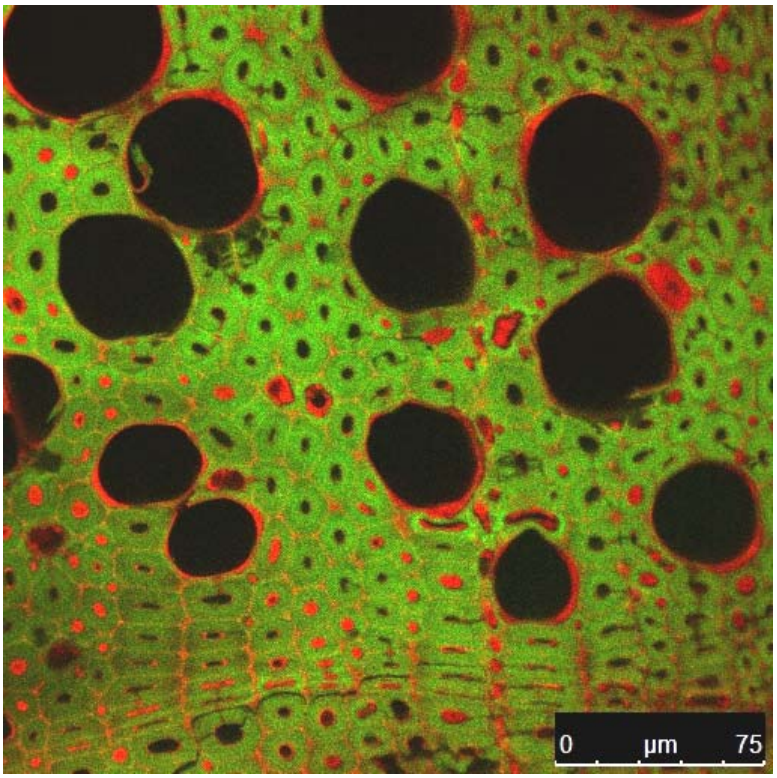


Fig 11 Fluorescence image of furfurylated beech (sample B40-1, FA 40, magnification 20x, zoom 2.87 x, image combining emission 500-520 nm and 690-720 nm)

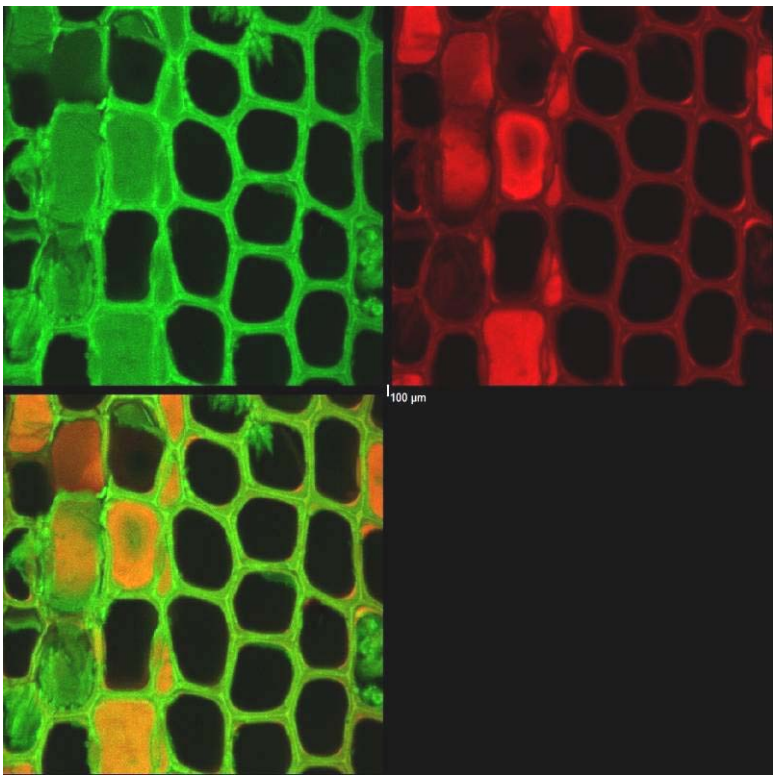


Fig 12 Fluorescence image of furfurylated Scots pine (sample J2, FA 40, magnification 40x, emission 500-520 nm upper left and 690-720 nm upper right image, below image combining both emission ranges)

5.1. Preliminary results

-samples, beech and Scots pine, seem to have slightly more fillings in the cell lumina than observed in samples described in chapter 4. However, the sample preparation and initial sample size used in treatment influence the results to an uncertain amount

-no change in emission of auto fluorescent and probably no change in chemistry can be seen compared to the samples that had bad results in marine tests

6. CLSM ON RESIN EMBEDDED FA-TREATED SCOTS PINE SAMPLES

Table 4 Overview over furfurylated and untreated Scots pine wood samples that were embedded

2-01FA	Scots pine FA, Low RoF[1]	
2-50 FA	Scots pine FA, high RoF	
2-50 K	Scots pine untreated	

[1] = ratio of filling

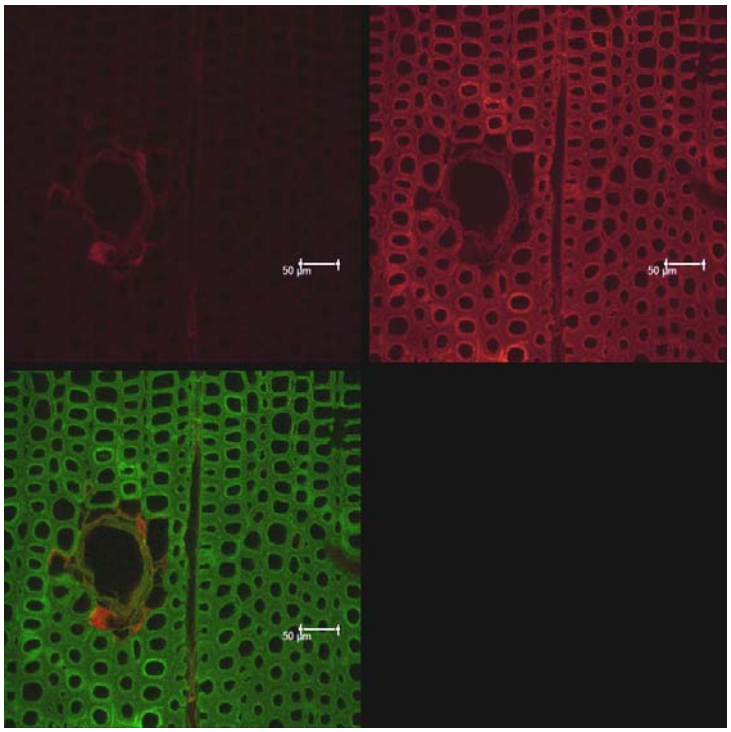


Fig 13 Fluorescence image of untreated Scots pine (sample 2-50K, 200 x magnification, zoom 1.64 x, emission 500-520 nm upper left and 700-720 upper right image, below image combining both emission ranges), the smart gain was increased up to ~ 1200V

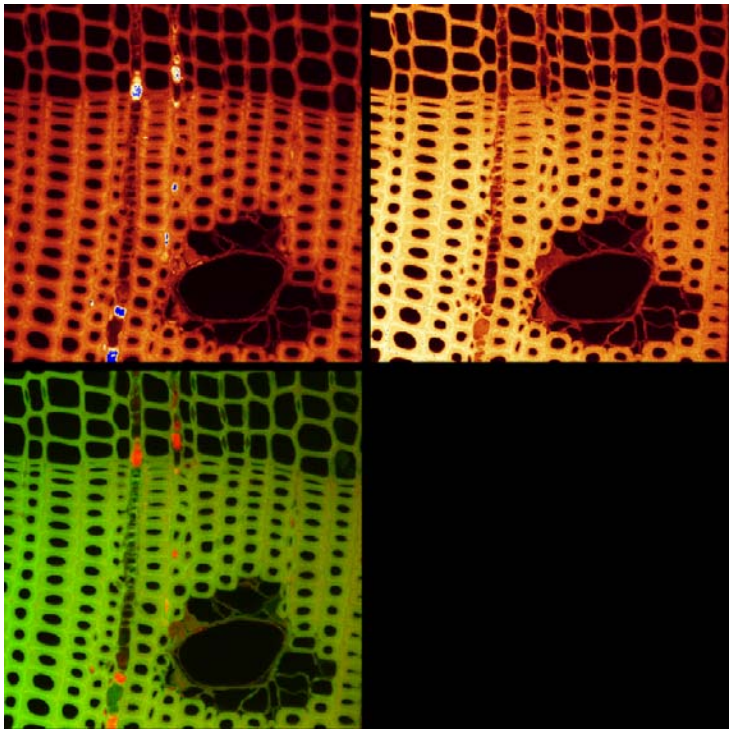


Fig 14 Fluorescence image of furfurylated Scots pine (sample 2-01FA, 200 x magnification, zoom 2.07 x, emission 500-520 nm upper left and 700-720 upper right image, below image combining both emission ranges)

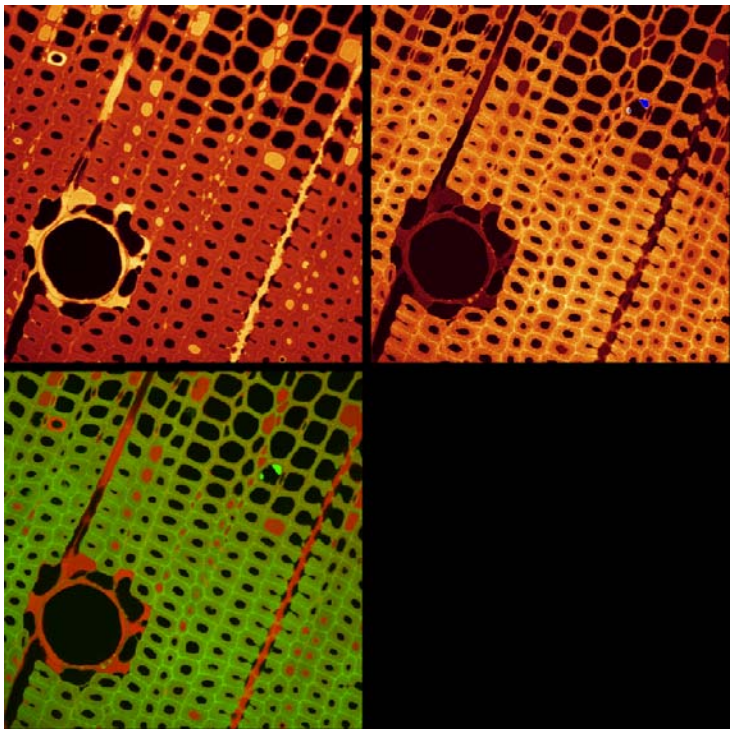


Fig 15 Fluorescence image of furfurylated Scots pine (sample 2-50FA, 200 x magnification, zoom 2.98 x, emission 500-520 nm upper left and 700-720 upper right image, below image combining both emission ranges)

6.1. Preliminary results

-the difference in uptake of furfurylated samples with high RoF and Low RoF can easily be seen in cross sections; higher RoF samples have FA fillings in the cell lumina

-uneven distribution of FA could also be seen in the images of low RoF samples

-untreated Scots pine and their emission due to auto fluorescence of lignin had as well as untreated beech samples insignificant influence on the emission images

7. CLSM ON FA-TREATED SAMPLES THAT UNDERWENT 4 DIFFERENT CURING PROCESSES

Main focus of this test was to analyse the influence of the curing process on the chemistry of the furfurylated material. 4 different curing processes were performed differing in time and temperature.

Table 5 different furfurylation treatments on Southern Yellow pine (FA 70):

treatment	temperature	Duration curing
A	103° C	5 hours
C	125° C	5 hours
F	103° C	25 hours
H	125° C	25 hours

The following parameters for CLSM were used:

Laser: Argon 488 nm 500-520 nm emission detector, smart gain 657 V

HeNe 633nm 690-700 nm emission detector, smart gain 736 V

7.1. Images of furfurylated samples of different treatments

7.1.1. TREATMENT A

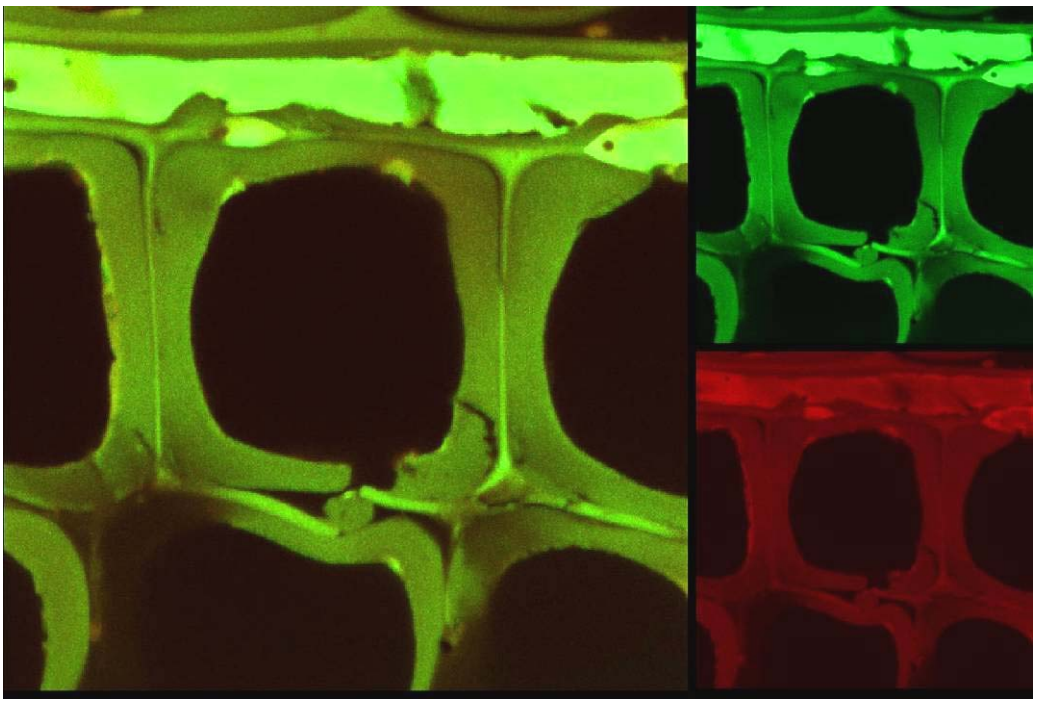


Fig 16 Fluorescence image of furfurylated Southern Yellow pine (FA 70, sample A, 630 x magnification oil objective, zoom 3.97 x)

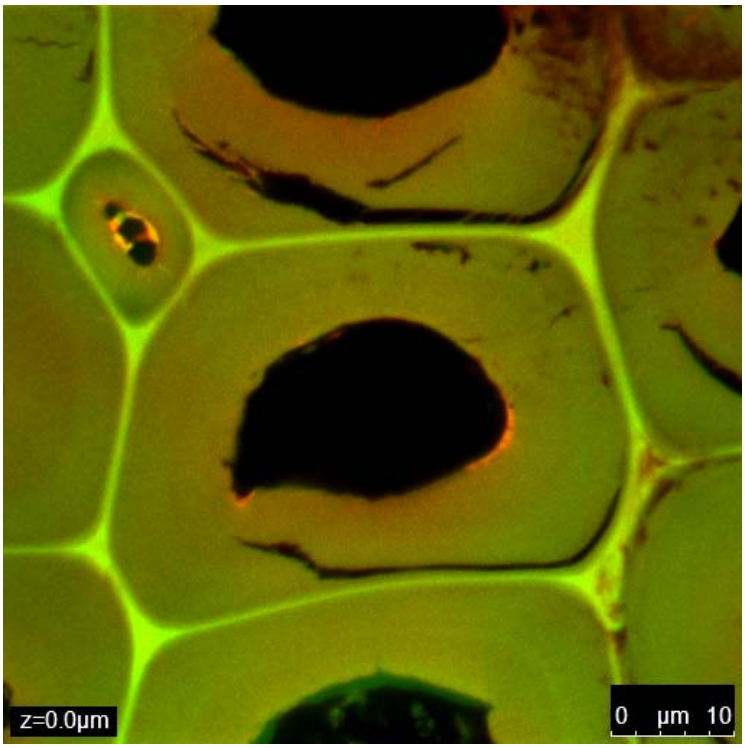


Fig 17 Fluorescence image of furfurylated Southern Yellow pine (FA 70, sample A, 630 x magnification oil objective, zoom 4.14 x)

7.1.2. TREATMENT C

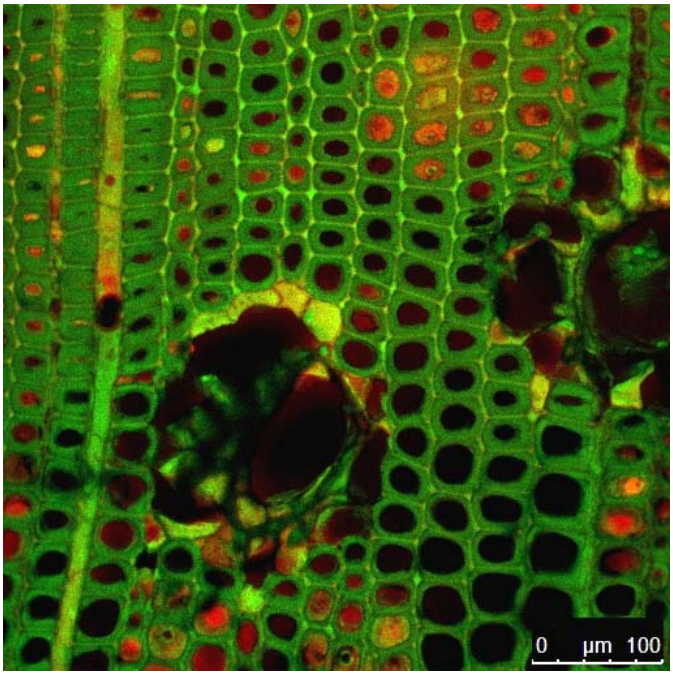


Fig 18 Fluorescence image of furfurylated Southern Yellow pine (FA 70, sample C, 100 x magnification, zoom 3 x)

7.1.3. TREATMENT F

Sample F was badly prepared and only a few images taken and therefore excluded.

7.1.4. TREATMENT H

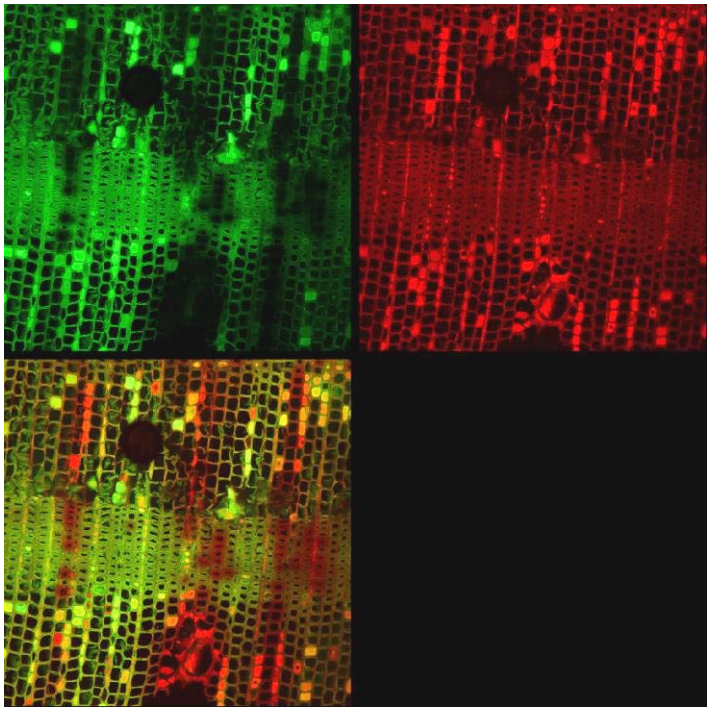


Fig 19 Fluorescence image of furfurylated Southern Yellow pine (FA 70, sample H, 100 x magnification, zoom 1.55 x)

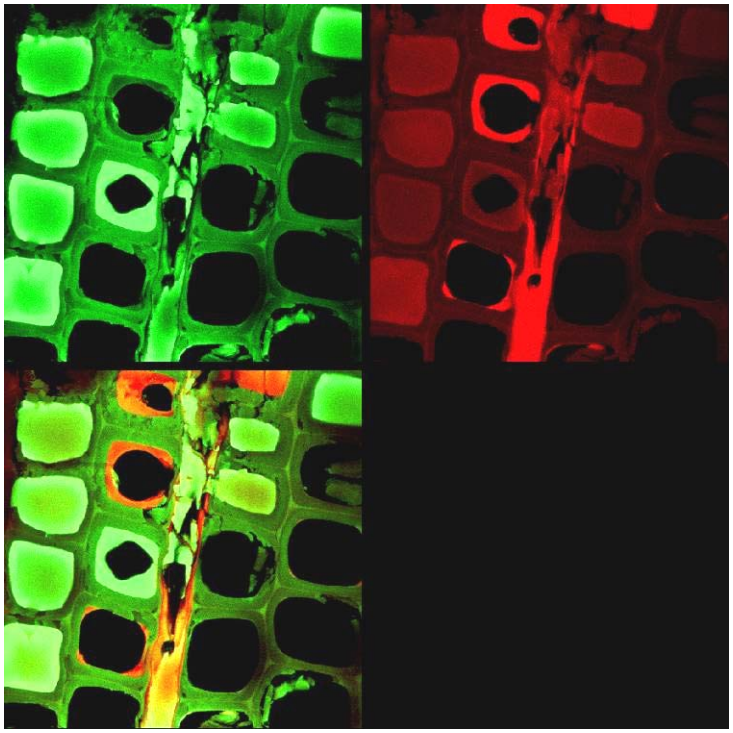


Fig 20 Fluorescence image of furfurylated Southern Yellow pine (FA 70, sample H, 630 x magnification, oil objective)

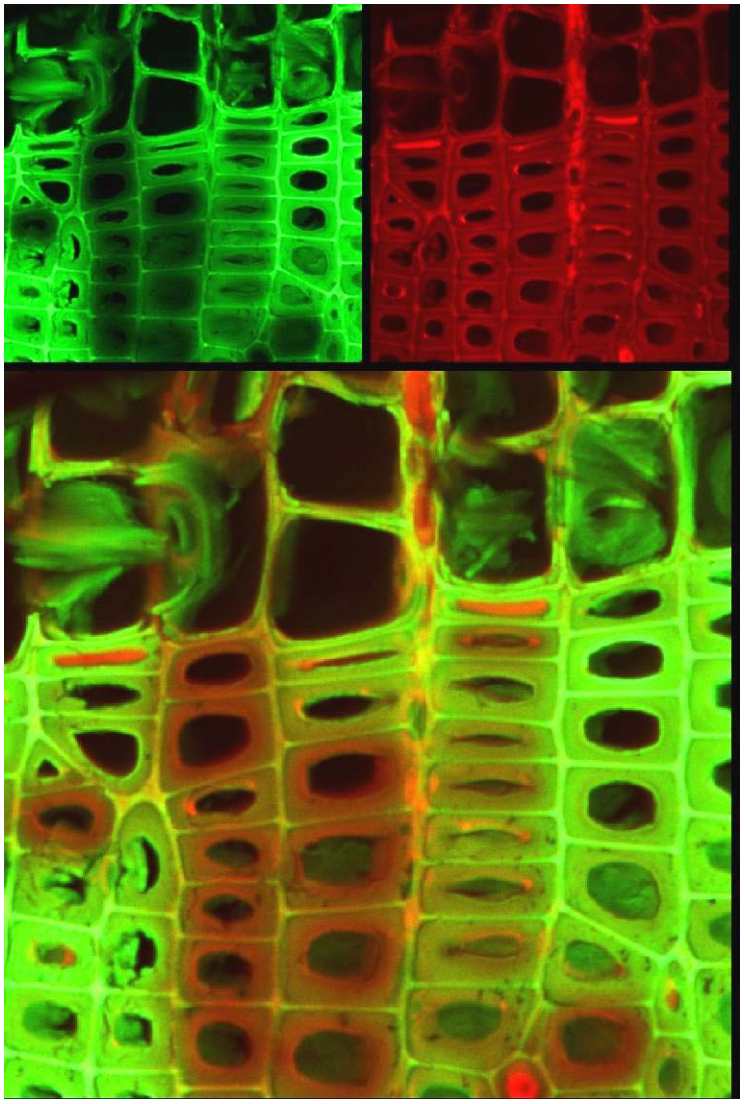


Fig 21 Fluorescence image of furfurylated Southern Yellow pine (FA 70, sample H, 400 x magnification oil objective)

7.2. Preliminary results

-sample H showed a high amount of “red” emissions (or less “green” emissions), which seems that the emission from 500-520 nm is low in some parts of the cross section

-the uneven emission could be caused by uneven moisture content of the samples (red areas higher moisture content?)

-the samples were not conditioned before and it can therefore not be excluded that the samples had different wood moisture contents

8. LASER SPECTROSCOPY LANDSCAPES, NIR AND FTIR

These tests were performed in Denmark at Skov & Landskab. Kontaktperson is Lisbeth Thygesen.

The samples described in chapter 7 were used for this analysis.

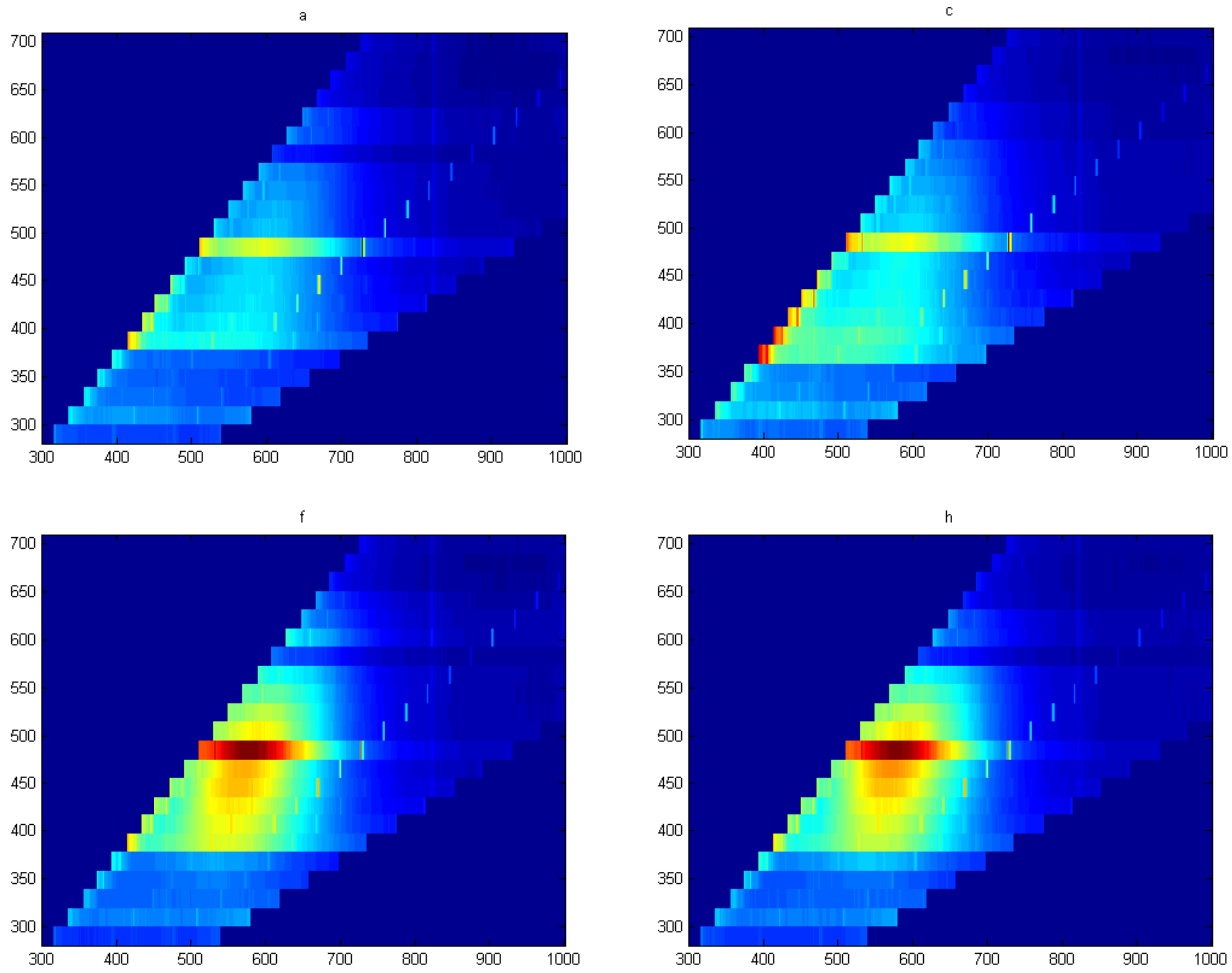


Fig 22 spectroscopic landscapes of different cured FA samples (Southern Yellow pine, FA 70)

The intensity of the emission of samples f and h is different (higher) compared to the intensity of samples a and c. However, differences in the emission wavelength can not be seen. No difference in the chemistry of this material is therefore assumed.

Results on FTIR- and NIR – studies on theses samples are available at Kebony ASA.

9. OUTLOOK

The factors that influence the chemistry of FA are pH-value, catalyst, amount of water, time and temperature. These factors can be changed and material can be produced with different chemistry and properties. If the chemistry is changed it is expected to change also the emission due to the auto fluorescent properties of FA.

Intensity of emission and emission range as well as area of emission in the sample were main focus in this study.

Some factors (such as sample preparation) that influence the outcome of this study were ignored. However, it is unsure if these factors influence the outcome of this study to a large amount.

If further tests are going to be performed, they should also take into account the following:

-a standard method for material and sample preparation should be preformed prior to all tests

-for preparation optimization and standardization of a preparation method both plastic embedded samples and cryotome prepared samples should be used in order to correlate these two methods and then use the best method

-the influence of long and strong radiation on the auto fluorescent properties - “bleaching” -should be investigated

-The influence of wood moisture content on the auto fluorescence of the sample material should be investigated

-tangential and radial sections should also be used additional to cross sections

It should be discussed whether this study should be expanded or continued. Lisbeth Thygesen came up with some ideas for further investigations:

1) A more thorough test of the use of fluorescence spectroscopy for finding a possible link between fluorescence spectra and the efficiency/success of the treatment - effect of surface characteristics (sawn/sanded) and which face (cross section, tangential, radial) is tested.

2) CLSM using the 'psuedo emission spectrum' possibility for different anatomical parts of the cross section (S2, middle lamella, cell corner) including good preparation of the samples prior to CLSM analysis

3) Explorative study where 'all' instruments available (including LF NMR) are tested on successfully and unsuccessfully furfurylated wood in order to try to identify a method that can tell the difference.

4) A bigger, more ambitious study (PhD?) on moisture in furfurylated wood - effect of WPG on the sorption isotherm, study of the states of water in furfurylated wood using LFNMR.

It is still unclear whether material and / or method of analysis (CLSM) is suitable in order to detect differences in chemistry of FA in furfurylated samples. However, from the performed test it can be concluded so far that only two different emission wavelengths with different intensities could be detected and no differences in chemistry of FA within the different material could be seen.