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Evaluation of different tissue de-paraffinization procedures for infrared spectral imaging

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Abstract:

In infrared spectral histopathology, paraffin embedded tissues are often de-paraffinized using chemical agents such as xylene and hexane. These chemicals are known to be toxic and the routine de-waxing procedure is time consuming. A comparative study was carried out to identify alternate de-paraffinization methods by using paraffin oil and electronic de-paraffinization (using a mathematical computer algorithm) and their effectiveness was compared to xylene and hexane. Sixteen adjacent tissue sections obtained from a single block of a normal colon tissue were de-paraffinized using xylene, hexane and paraffin oil (+ hexane wash) at five different time points each for comparison. One section was reserved unprocessed for electronic de-paraffinization based on a modified extended multiplicative signal correction (EMSC). IR imaging was carried out on these tissue sections. Coefficients based on the fit of a pure paraffin model to the IR images were then calculated to estimate the amount of paraffin remaining after processing. Results indicate that on average xylene removes more paraffin in comparison to hexane and paraffin oil although the differences were small. This makes paraffin oil, followed by a hexane wash, an interesting and less toxic alternative method of de-paraffinization. However, none of the chemical methods removed paraffin completely from the tissues at any given time point. Moreover, paraffin was removed more easily from the glandular regions than the connective tissue regions indicating a form of differential paraffin retention based on the histology. In such cases, the use of electronic de-paraffinization to neutralize such variances across different tissue regions might be considered. Moreover it is faster, reduces scatter artefacts by index matching and enables samples to be easily stored for further analysis if required.

1. Introduction:

Novel diagnostic methods are currently being sought in order to improve the efficiency of cancer diagnosis. Infrared (IR) spectroscopy is a promising candidate that has been demonstrated for potential application to the diagnosis of various cancers (1-9). IR spectroscopy provides complementary bio-molecular information from cells and tissues that can be translated into clinically exploitable information. In recent years, the number of applications based on IR spectral histopathology via large scale imaging of tissues has been increasing aided by technological advances in instrumentation capabilities (10-14). Although IR imaging is advancing, some important questions regarding the type of tissue- (frozen / paraffinized / de-paraffinized) and the various tissue processing steps that could be used for IR imaging still remain. Answering these questions is important if IR imaging technology is to be implemented as a diagnostic tool for pathologists. For IR imaging studies, frozen and chemically de-paraffinized tissues are most commonly used. Frozen tissues provide relatively pure bio-molecular information due to reduced tissue processing steps, although they have special storage requirements which limit large scale analysis and long term utility. Use of de-paraffinized tissues is advantageous because it provides access to large tissue banks that are pre-requisite for retrospective studies and to realize the capabilities of novel diagnostic methods. A de-paraffinization step has often been applied prior to IR imaging, due to the significant absorptions of paraffin in the mid-IR region, and is usually carried out using chemical de-waxing agents such as xylene or hexane. The paraffinized tissues are treated with de-waxing agents and progressively washed in decreasing concentrations of alcohol before passing through tap water. Although chemical de-waxing removes most of the paraffin from the tissue, the chemicals used in this process are known to be toxic (15).

Alternatively, de-paraffinization can be carried out electronically using mathematical algorithms which is faster, relatively less expensive, and most importantly avoids toxic chemical treatments (16-20). Electronic de-paraffinization also allows further measurement of the same section at a future date as it is non-destructive. Although both physical and electronic methods are available for de-paraffinization, no direct comparison of the advantages and limitations of the chemical and the electronic de-paraffinization methods on the same tissues has been made. Nevertheless, both methods have been shown to provide discrimination between diseased and normal tissues independently (3, 8). Recently, the use of paraffin oil as an alternate bio-friendly chemical agent for de-paraffinization has also been suggested. It has shown promising de-paraffinization results, in the process of high quality DNA

extraction from paraffinized tissues (21) and also in the process of histopathological analysis (22). Based on these encouraging results and considering the toxic nature of the commonly used chemical de-paraffinization agents, paraffin oil could be a potential alternative chemical agent for tissue de-paraffinization. However, its effectiveness has not been compared to the other available methods and moreover not been tested for IR imaging studies. In this regard, a comparative study has been carried out to explore the de-paraffinization capabilities of the commonly used chemical agents (xylene and hexane), bio-friendly paraffin oil (+ hexane wash), and electronic de-paraffinization. Paraffinized tissues were treated with xylene, hexane and paraffin oil at different time points and compared among themselves and then to electronic de-paraffinization method where no chemicals were used. The aims of this study were therefore to identify the most effective de-paraffinization method for IR imaging studies. It is hypothesized that if the alternate methods eliminate paraffin in comparable amounts to that of the standard methods (xylene and hexane), the use of toxic chemical agents may be avoided.

2. Materials and Methods:

2.1. De-paraffinization:

Sixteen tissue sections of 5 µm thickness were serially sectioned from a single block of paraffinized normal colon tissue with the approval of the Institutional Review Board. These unstained sections were placed on calcium fluoride (CaF_2) substrates and treated independently with different laboratory grade chemical agents at varying time points. The first five samples were treated with xylene (Fisher Scientific, UK), the second five with hexane (SpectrosoL®) and the last five with paraffin oil (Sigma-Aldrich, UK). For xylene and hexane treatments, time points of 15 min, 2 h, 6 h, 12 h and 24 h (Xylene T1 to T5, and Hexane T1 to T5) were followed. The chemical treatment was followed by washing the tissues in ethanol (Fisher Scientific, UK) for 1 min (2 changes), 95 % ethanol for 30 sec, and 75 % ethanol for 45 sec before washing in water. During the treatment, xylene and hexane were replaced every 3 to 4 h (for time points above 6 h). For paraffin oil treatment, time points of 30 min, 1 h, 2 h, 3 h and 4 h (Paraffin oil T1 to T5) were followed. The tissue sections were then washed in hexane twice to remove the paraffin oil, before passing them through ethanol baths similar to the former protocol. The experimental protocol of different de-paraffinization methods and the time points followed is represented in table 1. All the samples were then air dried for 24 hours before imaging. Section 16 was reserved for electronic de-

paraffinization and so no chemical treatment was applied. An additional tissue section was stained with haematoxylin and eosin as a histological reference (as shown in figure 1, left panel).

2.2. FT-IR imaging:

The same region of interest from all the chemically de-paraffinized tissue sections was imaged on a Perkin Elmer® 400 FT-IR imaging system equipped with a liquid nitrogen cooled 16-element linear array MCT detector. Measurements were carried out in transmission mode in the spectral range of 750-4000 cm^{-1}, at 4 cm^{-1} spectral resolution, acquired with 16 accumulations at a pixel size of 6.25x6.25 μm^2. The 16th unstained section was also placed on a CaF_2 substrate and imaged directly without any chemical de-paraffinization; electronic de-paraffinization (based on a modified EMSC) was instead carried out on this tissue post-imaging. The average image size measured was 685x553 μm^2 and consisted of ~9700 spectra. For each image, a background was recorded on an empty region of the CaF_2 substrate using the same parameters except for the number of accumulations which were increased to 120. Additionally, an IR image of pure paraffin region (4941 spectra) was also obtained from the 16th section, from the region outside the tissue where only paraffin was present. This was recorded in order to construct a paraffin model based on principal component analysis (PCA) to be used for electronic de-paraffinization and, for paraffin fit-analysis as described later in section 2.4.

2.3. Pre-processing:

The initial pre-processing step involved correcting all the IR spectral images for atmospheric water vapour and CO_2, using the built-in Perkin Elmer software. All of the following pre-processing and processing steps were carried out in the finger print region from 1000-1800 cm^{-1} using in-house programs written in Matlab R2012b® (Mathworks, USA). The IR spectral images were corrected independently using EMSC to account for scaling and offset effects using a polynomial baseline (4th order), a PCA model of pure paraffin spectra and a target spectrum. In this work the same target spectrum (also called the model spectrum or the reference spectrum) was used for all samples as described elsewhere (3, 17). The EMSC algorithm was also adapted to electronically de-paraffinize the IR image of the paraffinized tissue section as was demonstrated in previous studies (3, 17-20). In brief, the EMSC algorithm was developed initially to correct spectra from physical effects and to retain only chemical information from tissues (16). In the modified algorithm, EMSC is adapted in order to neutralize

the spectral contributions of paraffin using a modeling procedure. This basically consists of an interference matrix introduced into the EMSC algorithm which in this case is a paraffin model (consisting of the mean paraffin spectrum and the first ten principal components) accounting for the paraffin variability. Using this approach the variability arising from the paraffin is then neutralized across all the pixels in the dataset and only the chemical variability from cells and tissues is retained.

2.4. Paraffin fit-analysis:

The modified EMSC algorithm was used to perform fit analysis in order to find the remnant paraffin features across pixels in all of the IR images independently. For this, PCA was performed on the pure paraffin image. The mean spectrum of paraffin and the PCA model of the mean-centered paraffin spectra using the first 10 principal components (PCs) (shown in SI 1) were used to construct the model. For fit analysis, the fit coefficient for the mean paraffin spectrum was used to indicate the relative contribution of paraffin to each pixel in an image. Fit analysis for the IR image of the paraffinized tissue was performed both before and after subjecting the image to electronic de-paraffinization, to demonstrate the effect of the process. The median values and median absolute deviations (MAD) were calculated and plotted for each de-paraffinization method and across all time points for comparison. Median values were considered instead of mean values to avoid the influence of outliers (e.g. at tissue edges) on the average fit values.

3. Results and discussion:

To date IR imaging studies of tissues have mostly been performed using tissues that were chemically de-paraffinized. There is no standard chemical agent (the most common being xylene and hexane) and no standard time point under which the tissues are treated, and these parameters vary across different studies (1, 7, 12). There have been few studies where the efficiencies of the most commonly used chemical de-paraffinization methods were tested (15, 23). Recently, the efficiency of the less common paraffin oil in comparison to xylene was also carried out (22). Alternatively, the compatibility of electronic de-paraffinization has already been shown in previous studies (17). In order to investigate the most effective de-paraffinization method for IR imaging studies, different chemical agents (toxic and bio-friendly) and electronic de-paraffinization were tested over different time points. While a time point range of 15 min to 24h was used for xylene and hexane as this is the most common range used in previous

studies (1, 7, 12, 15, 23); for paraffin oil a range of 30 min to 4 h was used. The use of paraffin oil as a de-paraffinization agent was tested initially for use in DNA extractions (21) where an incubation time of only 20 min was used for dissolving paraffin. In another study (22) an incubation time of less than 5 min was used to de-wax tissues before analysing the staining efficiency. Considering these initial attempts, we hypothesized that a maximum of 4h would be sufficient to dissolve paraffin. In order to get the best comparative criteria and to avoid inter-individual variations, consecutive sections from one single tissue block were used. A representative white light image of the unstained section and the IR spectral image of a single section in comparison to the HE stained reference image is shown Figure 1.

The IR images were pre-processed and a fit-analysis was performed using the fit coefficients of the mean paraffin image spectrum calculated using an EMSC model applied to the data. Figure 2 shows the median value plots of the fit analysis with their median absolute deviation for the chemical de-paraffinization methods at different time points as well as the electronic de-paraffinization method. In figure 2, the T0 (zero hour) fit value is the reference which corresponds to the paraffinized tissue on which no de-paraffinization was performed and shows the highest fit value. The data points depicted in orange stars will be discussed later in this section.

Considering the chemical de-paraffinization methods independently, the average fit values for xylene showed a weak decreasing trend from T1 to T4 indicating continuous removal of paraffin with increasing time points. The T5 showed a deviation from this trend, however looking at the error bars this appears to be small. Although there was an increasing de-paraffinization trend from T1 to T4, the amount of time after T1 does not appear to be an important factor as most of the paraffin was removed in the first 15 minutes of xylene treatment in comparison to the zero hours (T0) (4 times approximately). This observation is in concordance with a recent study where initial 5 min of xylene treatment removed most of the paraffin and remained stabilized over the next increasing time points (23). A similar decreasing trend was observed for hexane from T1 to T4 on an average, but the rate of decrease between each time point was slightly reduced in comparison to xylene. It appears that the largest amount of paraffin that could be removed by hexane was removed at T1. Similar to that of xylene, T5 showed a small deviation but was even lower in this case. Comparing the average fit values of xylene and hexane, xylene appears to be a slightly better de-paraffinization agent in terms of the amount of paraffin removed and the time point required, although this difference is not significant due to the overlapping error bars.

A previous study however using Raman spectroscopy on a different tissue type observed hexane to be a slightly better de-paraffinization agent (15).

The paraffin oil de-paraffinization appeared to show an irregular trend particularly for T5. However comparing the average values of T1 and T4 there is a small increase in the de-paraffinization ability although not significant. The T5 of paraffin oil showed huge increase in the fit values which is close to that of the pure paraffinized tissue (T0), and was more pronounced than the previous two chemical agents. To further investigate this significantly deviating trend, a repetition was carried out for T5 of paraffin oil using time point T0 and electronic de-paraffinization as references. This was carried out using a paraffinized normal colon tissue section obtained from a different tissue block. It was hypothesized that this additional measurement to compare T0, T5 and electronic de-paraffinization for paraffin oil would be useful to verify if the initial T5 reading for paraffin oil was a reliable one or whether it had occurred erroneously. Therefore using the same instrumental parameters FTIR imaging was performed 1) directly on the paraffinized section and 2) on the same section post de-paraffinization by paraffin oil after 4hr incubation time (T5 time point). The same analysis was performed and three data points were generated i.e. 1) T0 (paraffinized), 2) Electronic (electronically de-paraffinized) and 3) T5 (de-paraffinized using paraffin oil). For direct comparison, these new data points were plotted (in orange stars) together with the same time points from previous readings in figure 2. Considering the new time points, it could be seen that the T0 and the electronic plots are consistent with the initial readings. However, the T5 values are present at a different position to what could have been expected if the time points of the regular paraffin oil trend was to be followed. Although this measurement is from a different tissue section, it does indicate that T5 paraffin oil removes paraffin to a similar extent as other time points with paraffin oil. Based on these readings, it is possible that the previous reading could have occurred erroneously during the experimental procedure. Since the new readings are from a different paraffin block, no direct conclusions could be drawn. Nonetheless in comparison to T0, T5 indicated paraffin removal with a median value closer to the other values obtained with paraffin oil with shorter time points. As with the previous chemicals, most of the paraffin that could be removed with paraffin oil appears to be removed within the first time point.

Comparing all three chemical agents, although the error bars overlap indicating similar de-paraffinization capabilities of all the chemical methods, the average median values of xylene are the lowest followed by hexane and then paraffin oil. The EMSC based electronic de-paraffinization plot (electronic)

corresponds to the paraffinized tissue but with electronic de-paraffinization applied. It has to be noted that in this case, the average fit value of 0 does not indicate that the paraffin is physically removed from the tissue rather the variance of paraffin signal across all the pixels has been neutralized electronically. This means that although the paraffin signal might be visible in the spectrum, it's influence is very much reduced when applying further multivariate algorithms such as linear discriminant analysis (LDA), which is commonly used to translate IR spectroscopic data into a diagnosis.

An important observation of this study is that irrespective of the chemical agent or the time point, although most of the paraffin was removed; there was still some paraffin remaining in the tissues, as can be seen from the positive fit values. This is however not visually apparent in the average spectra from each tissue (SI 2). It has to be noted that in the electronically de-paraffinized average spectrum in SI2, the variance originating from it is neutralized mathematically. Incomplete de-paraffinization was also observed in previous studies carried out on tissues from different organs using Raman spectroscopy (15, 24). Therefore, the influence of paraffin variability cannot be ruled out in cases where spectral information from such tissues is used to develop diagnostic algorithms. In this study, the first direct comparison of electronic de-paraffinization has been made with chemical methods and has been carried out on the same tissue block. Based on the fit values it could be observed that paraffin variability was still present in all the chemical methods which could be an influential factor in tissue discrimination or diagnosis.

For the first time, the ability of the less common paraffin oil as a chemical de-paraffinization agent has been tested and compared with the routinely used chemicals in pathology laboratories. Although, on average, paraffin oil removed less paraffin than xylene or hexane, the differences are relatively small. Weighing up the advantages of the paraffin oil over the toxic chemical agents (xylene and hexane) it appears to be an interesting alternate chemical de-paraffinization agent. Moreover, being a bio-friendly chemical, it can be easily discarded which makes it a good candidate for de-paraffinization in clinical practice. However, post treatment with paraffin oil the procedure does involve a quick wash with hexane. Therefore, although toxic chemicals are not completely avoided, the volume and time of usage is minimised.

Finally, all IR images were plotted based on the paraffin fit as depicted in figure 3. Images with positive intensities indicate higher fit therefore more remnant paraffin and *vice versa*. In addition to the incomplete paraffin removal when using all the chemical agents, one of the interesting features observed

was that the amount of paraffin removed from within the tissue varied with histology; namely glandular epithelial regions versus connective tissues. As can be seen in figure 3, the less dense connective tissue retained more paraffin across all images when compared to the glandular epithelial regions. A relative coefficient ratio between connective and epithelial tissue was then calculated to compare and to quantify the difference in paraffin retention. For this, K-means clustering was performed to isolate the epithelial and the connective tissue regions to choose their respective fit coefficients (data not shown). The coefficients were then offset such that the median coefficient for connective tissue was normalized to 1. The median connective tissue coefficient was then compared to the median coefficient from glandular epithelial regions as a ratio as shown in figure 4. The results were plotted for all the time points of the different chemical de-paraffinization methods. The ratio values of less than 1 across all the time points indicates that, after processing, paraffin is present in lower amounts in the glands than in the connective tissue. Comparing the ratio for xylene, hexane and paraffin oil, a trend is also observed where the ratio values are lesser for xylene followed by hexane and paraffin oil; i.e. xylene paraffin removal appeared to exhibit a greater tissue differential of remnant paraffin.

This is the first time a difference in paraffin retention has been reported within the same normal (colon) tissue and is probably based on the variation in tissue density between the glandular and stromal regions in this case. This observation also leads us to question whether discrimination using approaches such as PCA-LDA (18, 25) between tissue spectra may be made based on the affinity of paraffin to different histology types. In the current tissue analyzed, only two histological types are prominent (epithelium and connective tissue) and it has already been reported that malignant regions retain different amounts of paraffin from non-tumoral regions (24). As also discussed in the previous study (24), such observations bring out the question whether such differential affinity of paraffin could be an indicator of tissue pathology type as well and could be of diagnostic importance.

Chemical de-paraffinization using xylene or hexane is the most commonly used method. The fact that in these processes, paraffin still remains in the tissue suggests that the use of electronic de-paraffinization to neutralize the remaining paraffin variability should be considered as additional step. Alternatively, using only electronic de-paraffinization has already been shown to be a useful non-physical de-paraffinization method (3, 17). Moreover, having a gradient of retention across different histological types could mislead multivariate analysis. For example, if cluster analysis is performed on such tissues, the clusters may be assigned based on remaining paraffin densities rather than tissue

features. If this is the case, then electronic de-paraffinization would be a more reliable option since the paraffin variance is neutralized across all pixels. This can be observed in figure 3 where the electronically de-paraffinized tissue do not show any differential paraffin retention between different histology types as paraffin variance is same across all the pixels. This process could be applied on IR images obtained directly from paraffinized tissues or chemically de-paraffinized images. It is also important to consider that free and unbound lipids are leeched out during the process of tissue fixation (23) and prolonged treatments with chemical de-waxing agents may influence the cell lipid and protein content (26, 27). The electronic method therefore would minimize this effect by reducing the number of tissue processing steps. And since, the fit analysis is performed using a pure paraffin model, the variance from the lipid peaks (which have other distinct peaks in addition to those common to paraffin) is still retained in the analysis. However, using a model of pure paraffin means that potential interactions between the paraffin and the sample will not be accounted for. However we expect that the contribution of this interaction would be small in comparison to the variation of paraffin alone. Moreover, direct IR imaging of paraffinized tissues followed by electronic removal of paraffin contribution could remove problems related to scattering such as Mie scattering, as the refractive index across the sample is made to be more homogenous; thereby avoiding huge data treatment processes which may be required to correct for these issues. Furthermore, this is a quicker method, reduces toxic chemical usage and these tissues can still be archived for retrospective studies. However, the question whether electronic de-paraffinization is a more efficient option needs to be tested and validated on a larger sample set together with the chemical methods, in terms of changes in spectral characteristics and discrimination capabilities.

4. Conclusions:

The current study demonstrates that among the chemical de-paraffinization methods, on average xylene removes more paraffin followed by hexane and paraffin oil. However these differences are relatively small making paraffin oil an interesting alternative and bio-friendly chemical method. However, all the chemical methods used in this study not only fail to remove the paraffin completely, they also result in variable residual paraffin in connective and epithelial tissues. In such cases electronic de-paraffinization could be an effective additional method to neutralize paraffin variability across different tissue types.

However, electronic de-paraffinization can be employed directly on paraffinized tissues, thus avoiding any chemical treatment, which is faster, and additionally confers index matching thereby potentially reducing scattering artefacts.

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Table 1: Experimental setup showing the different de-paraffinization methods and the time points followed

Sample Number	De-paraffinization method	Designation	Time point
1	Xylene	T1	15 min
2		T2	2 hr
3		T3	6 hr
4		T4	12 hr
5		T5	24 hr
6	Hexane	T1	15 min
7		T2	2 hr
8		T3	6 hr
9		T4	12 hr
10		T5	24 hr
11	Paraffin oil	T1	30 min
12		T2	1 hr
13		T3	2 hr
14		T4	3 hr
15		T5	4 hr
16	Electronic	-	-

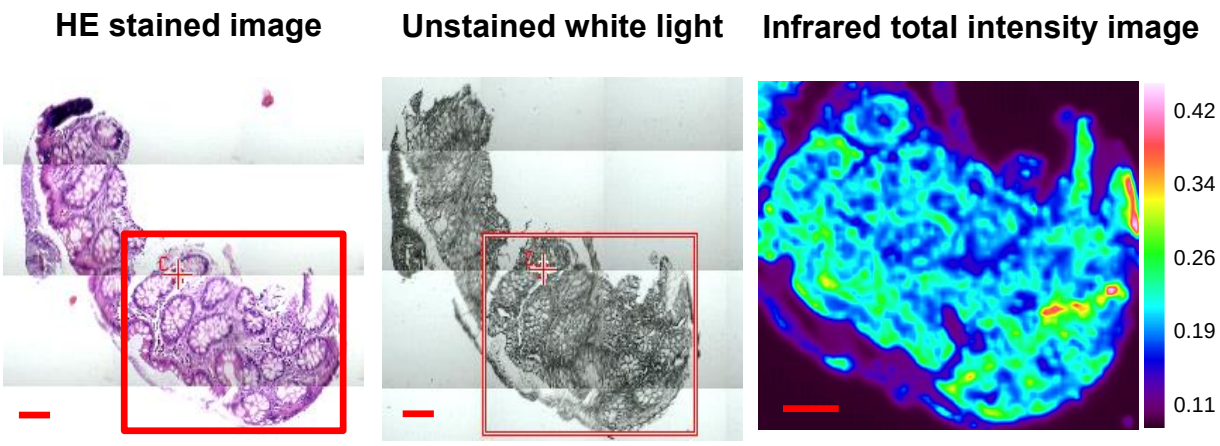


Figure 1: A representative unprocessed total absorbance infrared spectral image (right) obtained from an unstained tissue section (middle). The corresponding reference HE stained image is shown on the left. The measured region of interest is highlighted in the box. (Scale bar = 100 μm)

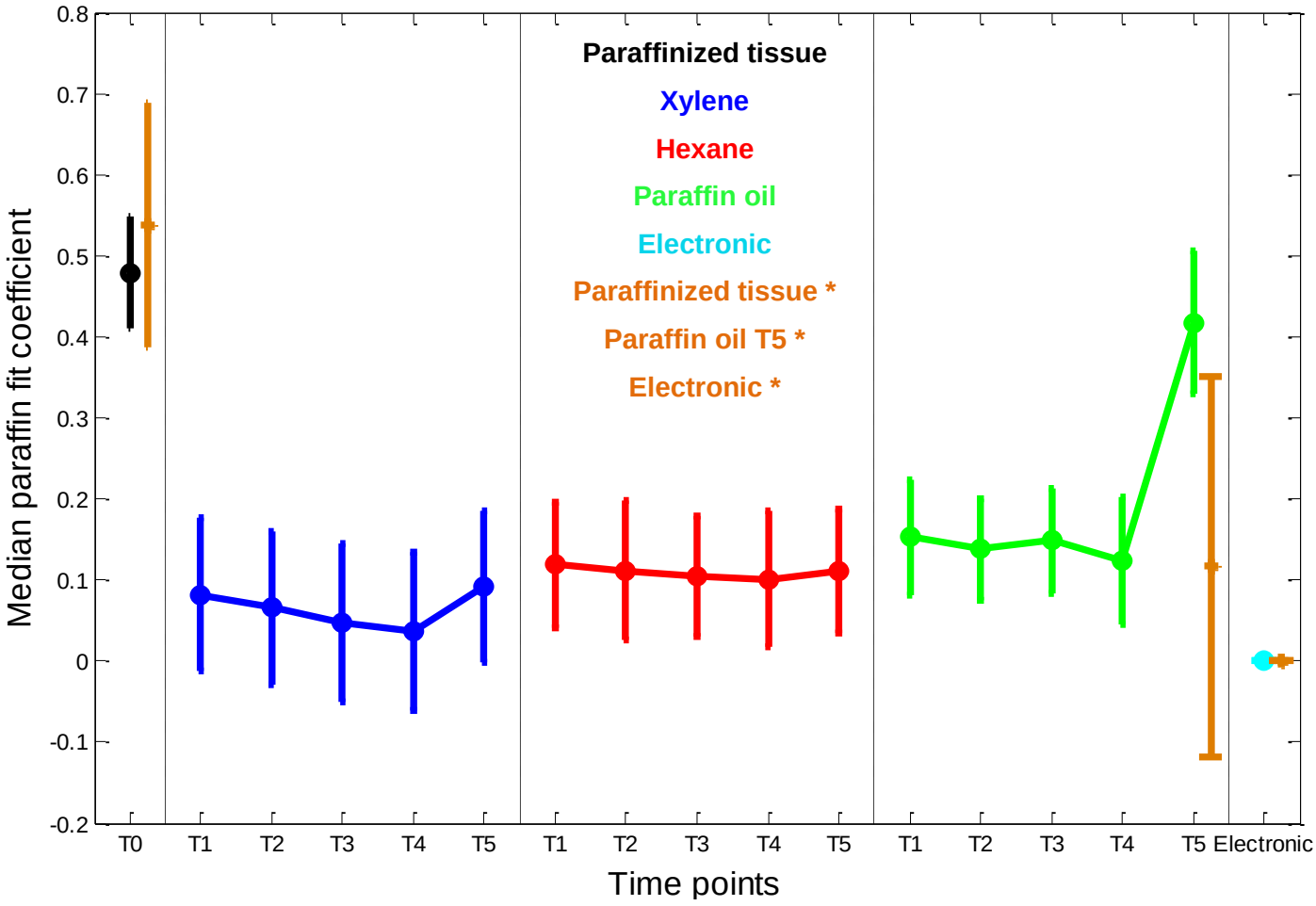


Figure 2: Median paraffin fit coefficients calculated over all tissue pixels in an image of a sample. All chemical de-paraffinization methods over five time points versus paraffinized tissue (0hrs) and electronically deparaffinised tissue are shown with respective color bars. Additional data points (in orange*) obtained from a different tissue section were used to validate the preliminary results.

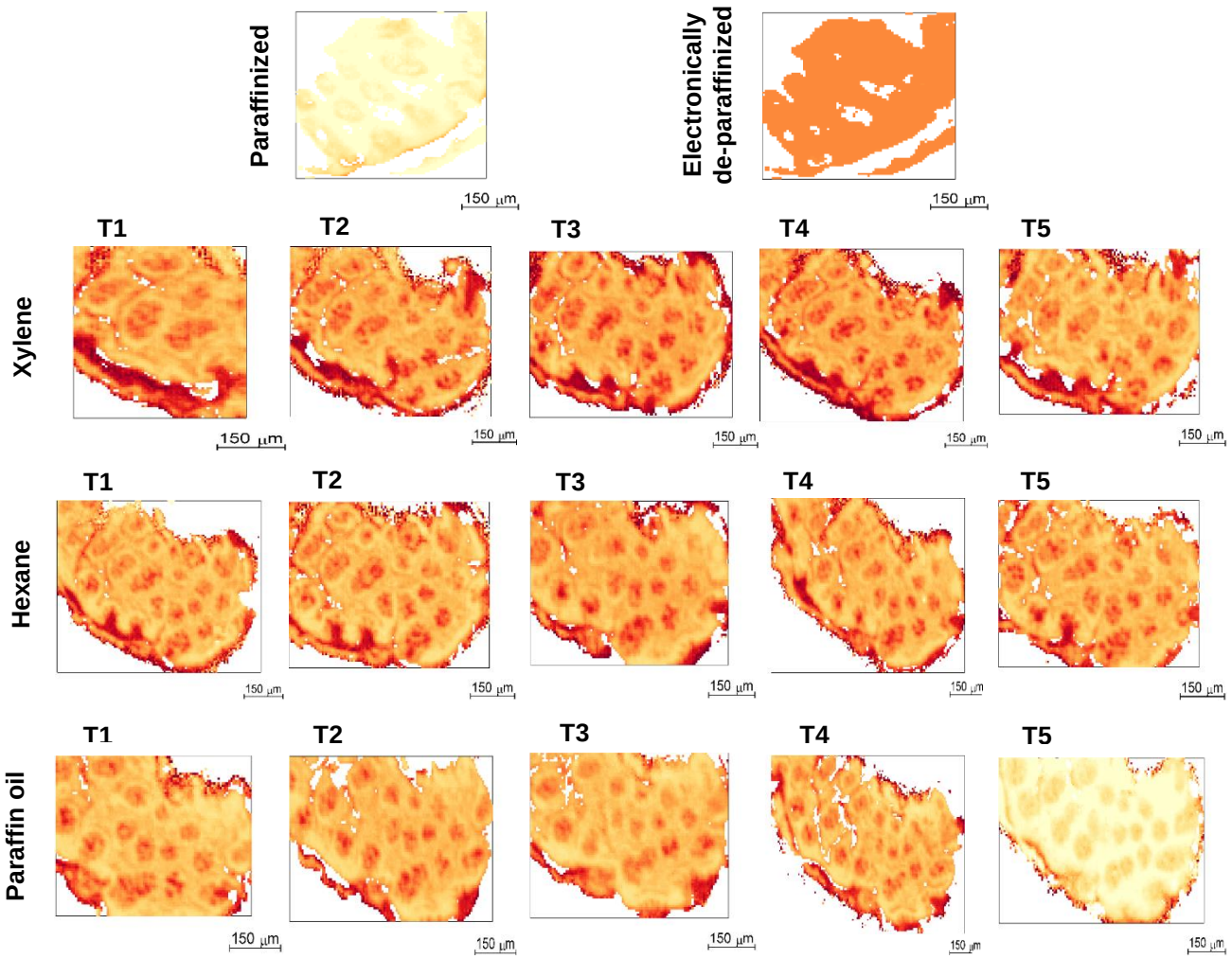


Figure 3: Intensity maps of the paraffin fit to all the de-paraffinization methods with their respective time points (T1 to T5). First row: Pure paraffinized tissue without any de-paraffinization and electronic de-paraffinization; Second row: Xylene de-paraffinization; Third row: Hexane de-paraffinization; Fourth row: Paraffin oil de-paraffinization. All the methods show differential retention of paraffin across all time points.

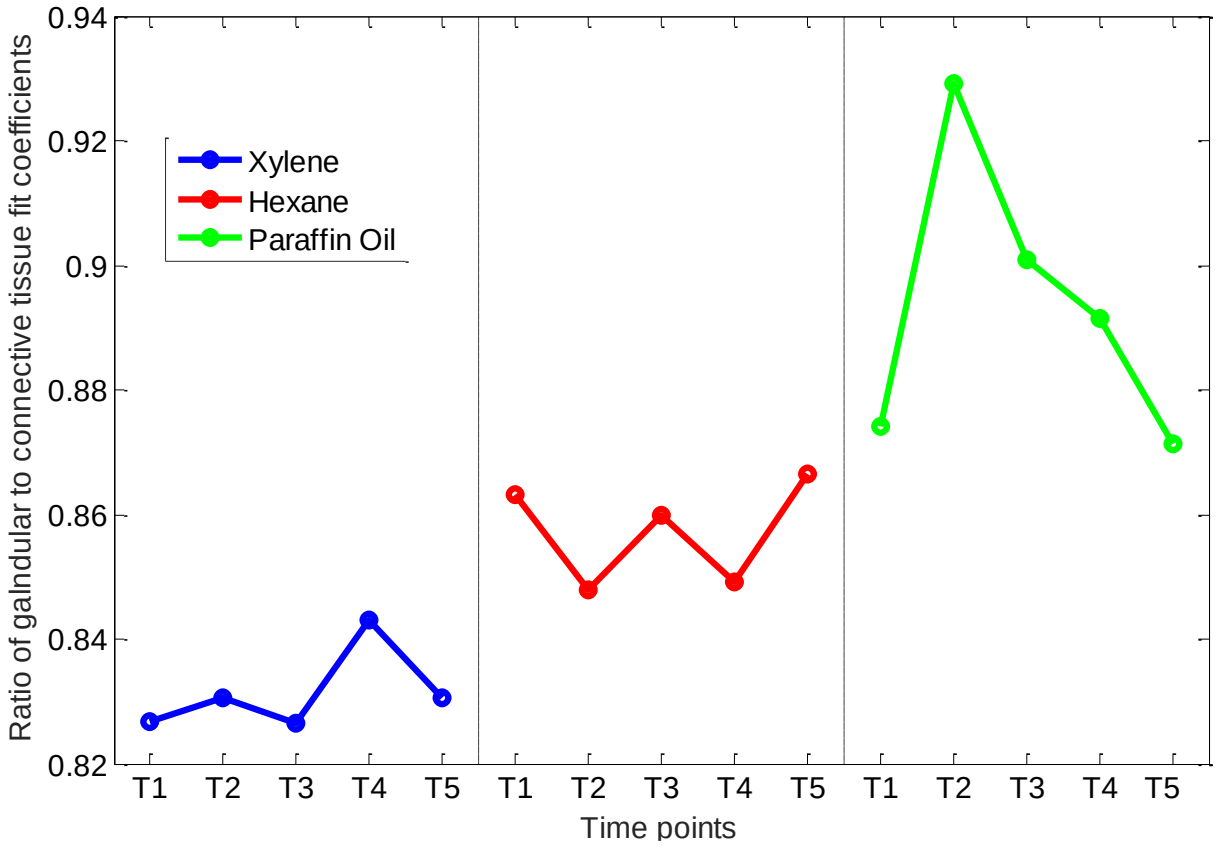
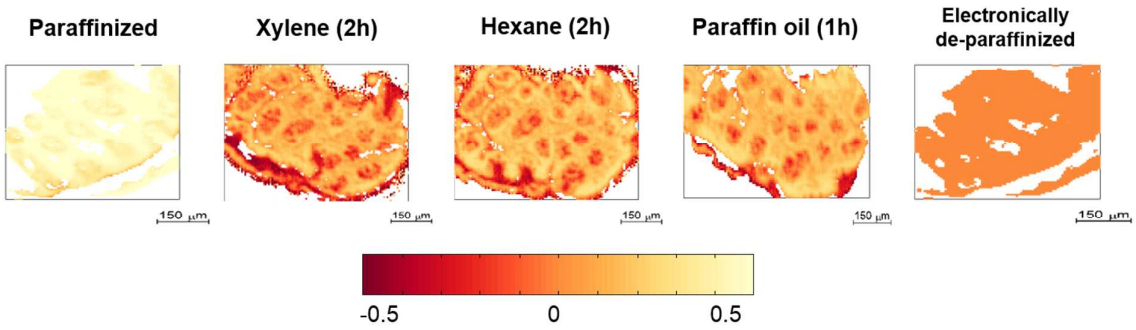


Figure 4: Ratio of fit coefficients of glandular regions to connective tissue (median offset to 1) for the chemical de-paraffinization methods at all the time points.



Differential distribution of paraffin in a normal colon tissue after various de-waxing procedures in comparison to a paraffinized tissue