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Research Article

Optimization of KI as X-Ray Computed Microtomography Contrast Agents for Murine and Chicken Epidermal Tissues Applications

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ABSTRACT

X-ray computed tomography (CT) is vastly used in many different applications in different fields such as materials science, medical science, entomology, anatomy, marine sciences. Since the X-ray is highly penetrable, 3D image of almost any material can be achieved by CT. The high quality image of the materials, which are composed of different types of atoms, can easily be achieved. However, obtaining the high quality images of the materials which has similar types of atoms or relatively soft structure becomes problem. Scientist investigating the soft tissues such as flesh, muscle, cartilage or animals in soft structure suffer from this problem. At this point, staining procedure, treating materials with contrast agents help researcher to enhance the image quality. In this work, optimization of KI based staining to obtain enhanced image quality in CT imaging of murine and chicken dermal tissues were studied. Results indicate that overstaining or staining the tissues in less concentration significantly affects the quality of the obtained CT images.

Key Words: *Computed Tomography, Skin, Phase Contrast Imaging, Absorption Imaging, Staining, Soft Tissue*

KI'nın Tavuk ve Kemirgen Epidermal Dokusunun Bilgisayarlı Tomografi Uygulamalarında Kontrast Ajanı Olarak Optimize Edilmesi

ÖZET

Bilgisayarlı Tomografi (CT ya da BT) malzeme bilimi, tıp, entomoloji, deniz bilimleri anatomi gibi birçok alanda değişik uygulamalarda kullanılmaktadır. X-ışınlara yüksek derecede penetrasyon (maddelerin içerisinden geçebilme) özelliğine sahip olduğundan hemen hemen bütün malzemelerin 3 boyutlu görüntüsü BT ile elde edilebilmektedir. Birden fazla materyal bir araya gelerek oluşmuş kompozit malzemelerin yüksek kalitede görüntüsü kolaylıkla elde edilebilmektedir. Ancak aynı tipte atomlardan oluşan malzemelerin ya da sertlikleri birbirine çok yakın olan yumuşak malzemelerin yüksek kaliteede görüntülerinin elde edilmesi zordur. Kıkırdak, kas dokusu gibi dokularla yumuşak yapıdaki hayvanları inceleyen bilim insanları yüksek kalitede görüntü alamama gibi probleme sıkça rastlamaktadır. Bu sorunu aşmak için lekeleme prosedürü yani incelenecek malzemeyi kontrast ajanına maruz bırakmak araştırmacıların yüksek kalitede görüntü elde etmesine yardımcı olmaktadır. Bu çalışmada KI temelli kontrat ajanının tavuk ve fare derisinin BT uygulamasında görüntü kalitesinin arttırılması amacı ile optimize edilmesi incelenmiştir. Elde edilen sonuçlar lekeleme prosedüründe aşırı ya da az KI uygulanmasının görüntü kalitesini düşürdüğü göstermektedir.

Anahtar Kelimeler: *Bilgisayarlı Tomografi, Deri, Faz Kontrast Görüntüleme, Soğurma Temelli Görüntüleme, Lekeleme, Yumuşak Doku*

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I. INTRODUCTION

Since the invention of X-ray imaging, it has used in many medical applications such as diagnostics. In addition, high permeability of X-rays attracted researchers in many fields like physics, engineering and material sciences to investigate their samples in detail and understand their physical and atomic structures [1]–[4]. The invention of X-ray tomography enables people to obtain the image of samples in three dimension (3D) with the help of the computer which therefore is called computed tomography (CT) [5]. CT found application in various discipline like medical sciences, biology, anatomy, taxonomy, entomology, marine sciences, materials science and engineering, geology etc [5]–[10] **.** In medical applications, CT was mostly used for diagnostic purposes. Since tomography used in the medical applications need to be fast and does not require very high resolution in micrometre (μm) scale, the devices used in this field were mostly focused obtaining image in 3D as fast as possible to reduce the X-ray exposition time [11], [12]. Despite the most actual purpose of tomography seems diagnostics in the medical applications, it has an important place in research both in biological sciences and in the non-biological applications. The necessity of detailed imaging in high resolution results in the development of tools which are able to scan in μm or nanometre voxel size precision and is called X-ray microtomography [13]

Researchers related to material science and engineering mostly uses the X-ray microtomography to investigate their samples in detail [13]. They are mostly investigating the composite materials where some important parameters like distribution, orientation, spread, alignment of two or more materials are very important parameters in the material properties [5]. Geologist are mostly focusing on the structure of their samples and distribution of the materials inside the rocks [14]. Availability of non-destructive investigation of the biological samples encourages researchers from different biological scientists used CT in their investigations [9]. For example, pollen, flowers, leaves, plants structures are widely investigated by plant scientists [15], [16]. Entomologists use CT for the investigation of insects' anatomy [9], [17], [18]. Anatomists use CT to understand the structure of certain organs or diseases or deformations occurs in the certain parts of human and/or animal body [19]–[22]. Embryologists find the opportunity to investigate the bird or reptile embryos in the without destruction by using CT [9], [23], [24]. Marine scientists use CT to investigate the certain parts of marine animals like fins, heads, brains, gills, suckers, shell structures [10], [25], [26]. The 3D information obtained from non-destructive investigations used in many applications and researches besides biological applications. Many researchers evaluate the structure of biological samples and tissues like shells, tendons, muscles to mimic their structures to develop more flexible, durable and cheaper materials [27]–[30]. In additions, many researchers assess and mathematically model the working parameters of biological tissues to develop artificial tissues and organs [29]. In all these applications, CT provides reliable and high-quality data in 3D.

Despite the advantages, there are some drawbacks encountered in the X-ray tomography applications. The contrast of the images, which obtained X-ray tomography scan, depends on density and atomic weight of samples scanned. Therefore, obtaining the high-quality image of biological samples in soft structures like soft tissues, muscles, tendons with high contrast is problematic [31]. Certain protocols like staining and stabilization of samples need to be applied prior to the CT scan. In the staining procedure, the samples were dipped in special contrast agents like KI, IKI (Lugol's solution), Phosphotungstic Acid (PTA), Phosphomolybdic Acid (PMA), gold nanoparticles and bismuth nanoparticles for certain duration depending on desired staining procedure [8], [19], [32]. To stabilize the sample, it was embedded in some soft polymers like epoxy, resin or agarose. That protocols

prevent biological samples to decompose, help to reduce the vibration, motion, noise and increase the contrast of the image.

As far as the knowledge of the author, the first time in the literature that the structure of the skin was investigated by using X-ray tomography technique. Previously various imaging and tomographic techniques were used to assess and to investigate the structure of the skin such as fluorescence microscopy [33], [34], photoacoustic tomography [35], [36], optic microscopy [37], [38], optical coherence tomography [39], electron microscopy [38], [40] and atomic force microscopy [41], [42]. Other tomographic techniques (such as optoacoustic tomography) applied to the skin could not give structure details in 3D as high quality as X-ray tomography did. Moreover, the skin data obtained from microscopic techniques gives the structure of skin in 2D. In this report, step by step application of protocols to increase the image quality and image contrast was evaluated for chicken and mouse skin for X-ray microtomography. Effect of sample stabilization and staining protocols on sustaining images in high quality were illustrated. It was believed that the protocols provided in this report may help researchers investigating soft tissues to achieve better contrast in great details. Obtaining high quality images of soft tissues in 3D images helps many researchers to deeply understand the structures of soft tissues like skin. In addition, high quality tomography data of skin which were obtained via using CT could be used in computer simulations, computational works, biomimetic, materials engineering, and 3D and 4D printer applications. As this investigation may help researchers to understand the structure of skin better in medical sciences, it may also help researchers to develop more durable and flexible materials that can be applied to many technologic and medical implications.

II. MATERIALS AND METHODS

A. OBTAİNİNG THE BİOLOGİCAL SAMPLES

Two different samples were used in the assessment: chicken skin(*i*) sample and mouse skin(*ii*) sample. The chicken skin sample was obtained from a commercially available chicken. Hairless skin belongs to the chicken leg of skin was taken commercially available chicken which was sold in supermarkets. Using the skin of commercially available chicken request no permission to be used in the experiments and it is also cost effective. It provides numerous advantages for prior investigations.

C57BL/6 mice were chosen as suitable candidate as a source for mouse skin investigations. C57BL/6 mice were used in many biological experiments since the organ structures and metabolism of these species quite similar to humans. They are very reproductive and cheap compared to other experimental animals. Since the skin structure of mouse was quite like human, investigation of mice skin as healthy skin will give similar results that can be obtained from human. In addition, ethical procedures followed in the animal experiments request less effort than human originated biological samples.
In the sacrifice of animals and sample preparation processes, UK animal sacrifice regulations were followed [41]. Animal sacrifice processes and tissue obtaining processes were performed by licensed professionals in accordance with United Kingdom Home Office regulations (Animal Scientific Procedures Act [1986] (license PPL 70/7166)) [41]. In our mouse skin samples, 6 months old C57BL/6 mice tail skins were used. The animal tails were obtained from the University of Portsmouth Animal Hospital where mice used in the experiments were sacrificed by the CO_2 application. In the sacrifice procedure, mice were kept in a special box oxygen level was decreased gradually and the CO_2 level increased until the mice dead then the tails were dissected from the animal body. Skin was

peeled away from the tail using forceps to isolate the full skin thickness and allow further sample preparation [43]. Dissected tails then skinned and cut in small pieces and each piece put separate Eppendorf tubes. Tubes are then labelled and frozen to eliminate the decomposition of biological samples. Before the sample preparation, the required amount of skin was taken from the freezer and thawed.

B. STAINING

Staining is a special procedure followed in the tomography application to enhance the contrast and quality of obtained images. In the staining procedure, the samples were dipped in special contrast agents for a certain duration depending on desired staining procedure. KI, IKI (Lugol's solution), Phosphotungstic Acid (PTA), Phosphomolybdic Acid (PMA), gold nanoparticles and bismuth nanoparticles were some examples of contrast agents which were widely used in this field. Before the staining procedures, some extra applications may be followed to prevent samples from decomposing [44]. Such processes are called the fixation process. Keeping biological tissues in formalin or ethyl alcohol is well-known fixation processes that cause biological tissues to dehydrate [8]. Such dehydration protects biological samples from decomposing [4].

In our investigations, our group avoided from dehydration protocols since dehydration of biological samples may result in alterations in the structure and mechanical properties of the skin. In addition, it is well-known fact that fixation processes may cause shrinking or structural deformations of biological samples [8][32], [45]. Our first attempt was trying to optimize tomographic imaging protocols of the skin without fixation and staining to obtain detailed tomographic images of skin tissue. In our work, IKI solution was used in the staining process. IKI solution in various concentration was prepared. Skin samples were plunged in the IKI solution. Plunged skin samples were kept in the IKI solution for various durations depending on the application. When the skins were kept there enough, skins were taken out of the IKI solution. After the staining process, samples were cleaned with pure water and were put in Eppendorf tube. The samples either directly visualized using tomography or they stabilized in Agarose then tomographic imaging was applied. Since agarose is an organic molecule with good humidity level, it keeps samples humid. Moreover, X-ray attenuation of agarose is low and viscosity of the agarose helps samples to stay stable.

C. SAMPLE STABILIZATION

Zeiss Xradia Versa X-ray computed Microtomography was used in the tomographic imaging process. The system is very powerful and enables to produce tomographic images up to a couple of hundred nm voxel size. To obtain images in such high resolution requires prolonged exposition times. In the tomography imaging process, 800 to 2000 X-ray images of samples were taken in 360 degrees to obtain the reconstruction of the sample in 3D. Depending on desired voxel size (resolution), intensity and image quality, each image requires certain X-ray exposition time. Increased exposition times results in the increased duration of the measurement. Depending on the investigation and material type a single measurement might take up to 20 hours. It was known that biological samples are part of living organisms and sustain their lives as part of the biological organism. The biological organisms provide the conditions to keep them alive. Biological organisms separated from their main body parts start to die and decompose. Therefore, biological samples are fragile to external effects such as temperature, humidity, oxygen rate etc. The working conditions of CT machines were mostly quite similar to atmospheric conditions.

For the skin sample measurement, room temperature brings some drawbacks. The prolonged measurement durations cause some alterations in the properties of the skin that requires intensive care. For example, exposing skin to atmospheric room temperature conditions results in dehydration of the skin which causes an alteration in the size and mechanical properties. To stop this case biological samples were scanned in closed tubes or Eppendorf capsules to stop skin dehydration.

Gravity also plays important role in the instability of samples. To obtain good thin side view skin samples were mostly placed on perpendicular inside the tubes or Eppendorf capsules. The CT device took about between 800 to 2000 images in 360 degrees (depending on desired measurement properties). To provide a perfect reconstructed image, the device re-calibrates itself with taking an image in a certain position. The calibration process can be repeated more than 100 times in the scanning. Therefore, all the movements in the scanning process cause vibration and shake on the tube. The shake movements cause the sample to slide down with the help of gravity. The downslide decreases the quality and distorts the reconstructed 3D image. We tried to attach the sample to the wall of the tube with a pin, but it did not give a very good solution. Our group then found a solution by embedding skin within 1% agarose solution. Agarose is a wax-like polymer; it is liquid when it is hot and gets viscous when it gets cold. In our applications, the tube was filled with agarose solution after the placement of skin, the solution gets hard and stabilizes the skin inside the tube and remains the same. The application provides positional and biological stability. 1% agarose solution was used to stabilize the skin samples. 1% agarose was solved in distilled water using a microwave oven. When the water boiled, agarose powder was totally dissolved in the water. Then, the solution was left for cooling. When the agarose solution becomes viscous, it was poured into the polymer tube using a pipette and skin samples were placed inside the tube.

D. IMAGE PROCESSING

MeVisLab software was used in the image processing. MeVisLab is an open ware that which was developed by MeVis Research GmbH (which became Fraunhofer MEVIS in 2009). The software was developed for the medical imaging purposes. The development process of the software was begun in 1993. The software has been updating regularly. The software can visualize any kind of tomographic data. In the image processing, raw X-ray tomography files were obtained. Depending on the resolution of the image and the number of images taken in the tomography imaging, the size of the folder may be between 800 MB and 3 GB. The raw tomography folder was transferred to the MeVisLab. The software enables researcher to construct tomography images in 3D using special tools and modules. Software uses tools which is defined by DICOM (Digital Imaging and Communication in Medicine) standards. Processed image can be visualized in slice based. For example, software enable researcher to slice images horizontally and vertically. In figure 1, 2, 3, 4 and 5, 6 and 7, horizontal slicing method was used where random slices from Eppendorf tube was chosen. Digital image adjustment was applied using contrast and brightness tools. Brightness and contrast of the image was enhanced to obtain better resolution. Threshold tool was used to ignore the details in the images where certain details under a threshold point was removed by program. As a results clear images were obtained. 3D images were performed using 3D module of the software. To remove the agarose around the skin threshold tool was used. Brightness of the agarose was defined, and threshold of agarose brightness was applied where an image which was removed from agarose was obtained.

III. RESULTS AND DISCUSSION

A. STABILITY

As it was discussed in the previous section, in the imaging process the sample holder changes it position hundreds of times. Exposition of the sample to the ambient conditions result in decomposition or dryness that can change the structure of the living organism. Both motion of sample holder and the exposition of sample to external factors result in deterioration in image quality and on the sample itself. In this section, problems encountered imaging of skin and practical solutions to overcome these problems were discussed.

Since mouse tails used in the experiments were costly and requires special regulation, for stability research, chicken skin (a cheap and reliable source) was also used in our prior investigations. Chicken skin is quite similar to mouse skin but has more fibrous and fatty tissues. The hair-free chicken skin was obtained from the leg of commercial chicken. A polymer tub in 5 mm diameter was cut in 3 cm long and placed on the CT sample holder. The chicken skin was cut and placed inside the polymer tube without any support. The top of the tube was sealed with parafilm. CT scans were performed in 40 KV with varying exposure times. Both absorption and phase contrast imaging were performed. No support was put inside the tube. As a result, topographical images of skin were obtained (See Figure 1). Figure 1 shows the absorption tomographic images of chicken skin showing an up to down look image. It was seen that detailed visualization of epidermis and dermis could not be obtained. Fatty and fibrous tissue and a region belong dermis and epidermis could be identified but a sharp separation between dermis and epidermis could not be observed. In addition, distortion in the image could be seen which occurs due to shakes or decomposition. Such motions refrain us from observing sharp images. Especially at the edges of images distortions were found stronger that image looks unfocused and blurred (see Figure 1c). Overall image quality was found poor and details belong to important parts of the skin were unidentifiable.

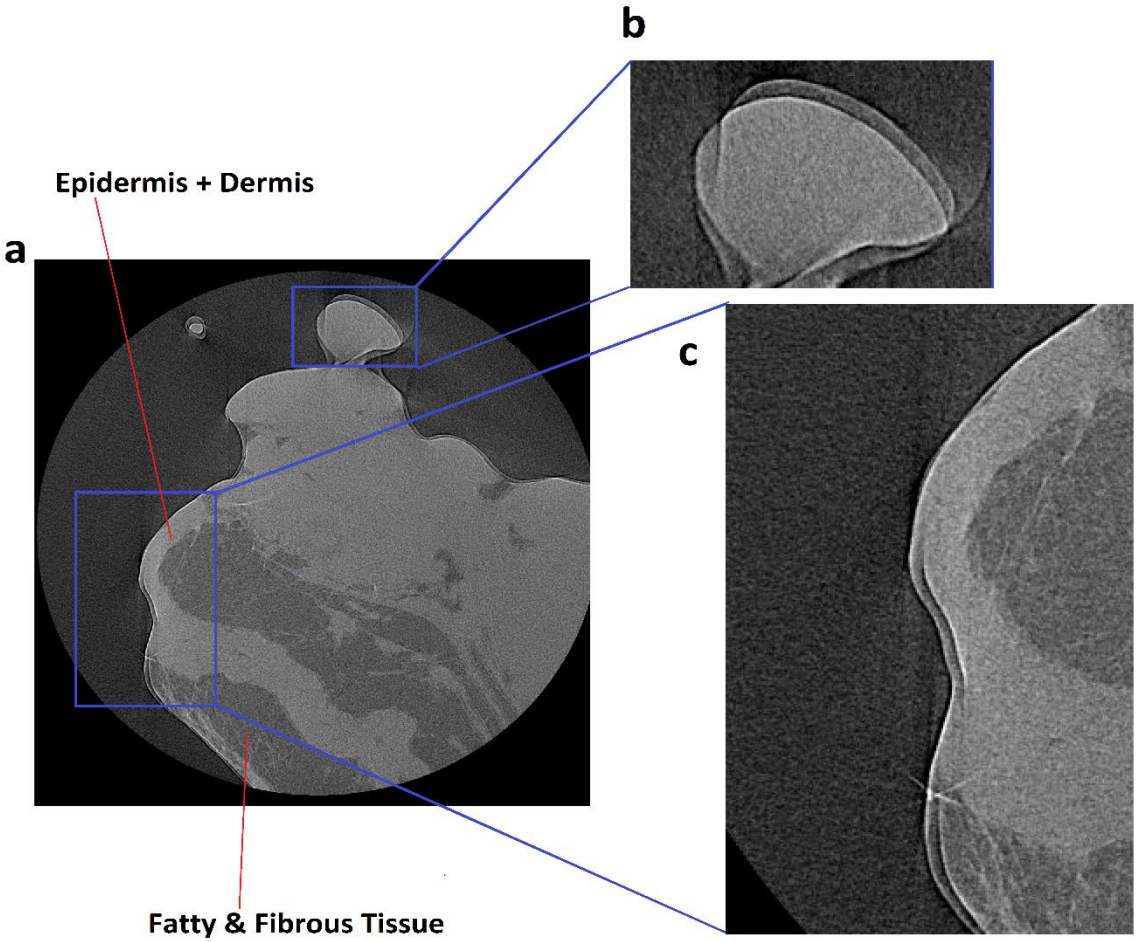

Figure 1. (a) *The absorption tomography image showing an up to the down look of the chicken image.* ***(b)*** *Zoomed region where a huge amount of shift occurs.* ***(c)*** *Zoomed region where the shift at the edges of the image could be identified.*

Mouse skin was also evaluated using the same parameters. The skin was placed in a polymer tube and the top of the tube was covered with parafilm. For the visualization of mouse both absorption imaging and phase contrast imaging were performed. The results were found parallel to that of results obtained from the chicken sample. Even though mouse skin sample was thinner than chicken sample instability in both phase contrast imaging and absorption imaging could be seen (See Figure 2). Phase contrast images obtained from mouse skin was found highly unstable as can be seen in Figure 2 a, b, and c. Shifts and shakes in the image could be identified (See Figure 2c). Movement in the sample during tomography imaging result in failing that even tube in which sample was kept look shifted as well. Despite the shake movement, phase contrast image gives detailed visualization. Hair follicles and hairs could be identified (see Figure 2c) but a clear separation between the epidermis and dermis could not be observed. Images related to absorption tomography were presented in Figure 2 d and e. Samples look sharper in absorption images that indicates slightly less movement of mouse skin. All in all, small shifts at the edges of the skin could be seen. The images obtained from absorption tomography does

not give information about the detailed structure of mouse skin. Only hair could be identified from images, but it is hard to identify hair follicles. It is also difficult to distinguish epidermis from dermis.

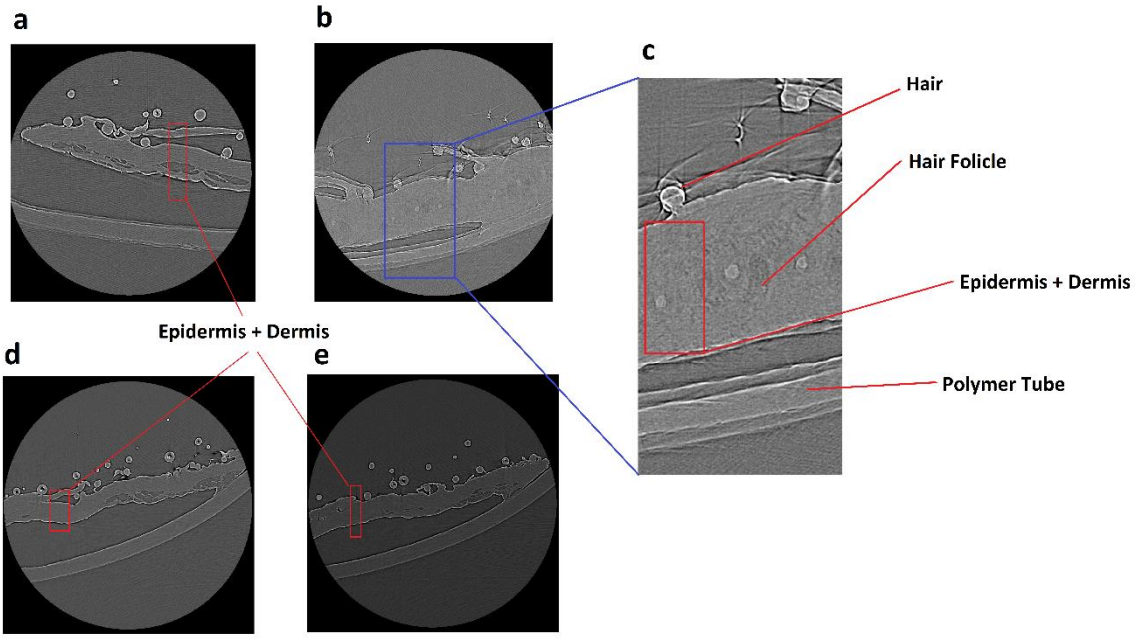

Figure 2. *Phase contrast tomography (**a, b, c**) and absorption tomography images (**d, e**) of mouse skin from top to the down look were presented.*

Since the tomography images obtained from unsupported samples was found unstable, samples were stabilized using agarose. 1% agarose solution was used to stabilize the skin samples. 1% agarose was solved in distilled water using a microwave oven. When the water boiled, agarose powder was totally dissolved in the water. Then, the solution was left for cooling. When the agarose solution becomes viscous, it was poured into the polymer tube using a pipette and skin samples were placed inside the tube. The top of the tube was then sealed with parafilm. The procedure was performed in the stabilization of both chicken sin sample and mouse skin sample.

Chicken skin sample which stabilized with agarose is scanned by both absorption tomography imaging and phase contrast tomography imaging. The results belong to the chicken sample was presented in Figure 3. Absorption (Figure 3 a and b) and phase contrast (Figure 3 c, d and e) scans result in slightly more stable images. Both images obtained from absorption scan and images obtained from phase contrast scan do not show shifts originated from the vibration or motion of the sample. It was concluded that the agarose solution could hold the skin sample stable. Images obtained from absorption imaging illustrates the fatty and fibrous tissues in great details. Dermis and epidermis look intact, but it is hard to distinguish the epidermis and dermis section from each other as this section find blurred. As agarose solution surrounds the skin sample, it is difficult to distinguish agarose solution from skin itself as well. In the phase contrast imaging fatty and fibrous tissue could be seen in great details. Skin sample and agarose could be distinguished. Swirls and wrinkles in the epidermis and dermis region could be identified. Multi-layered structure of the epidermis and dermis could be identified but the detailed structure could not be seen. The quality of phase contrast images was found higher than the quality of absorption images.

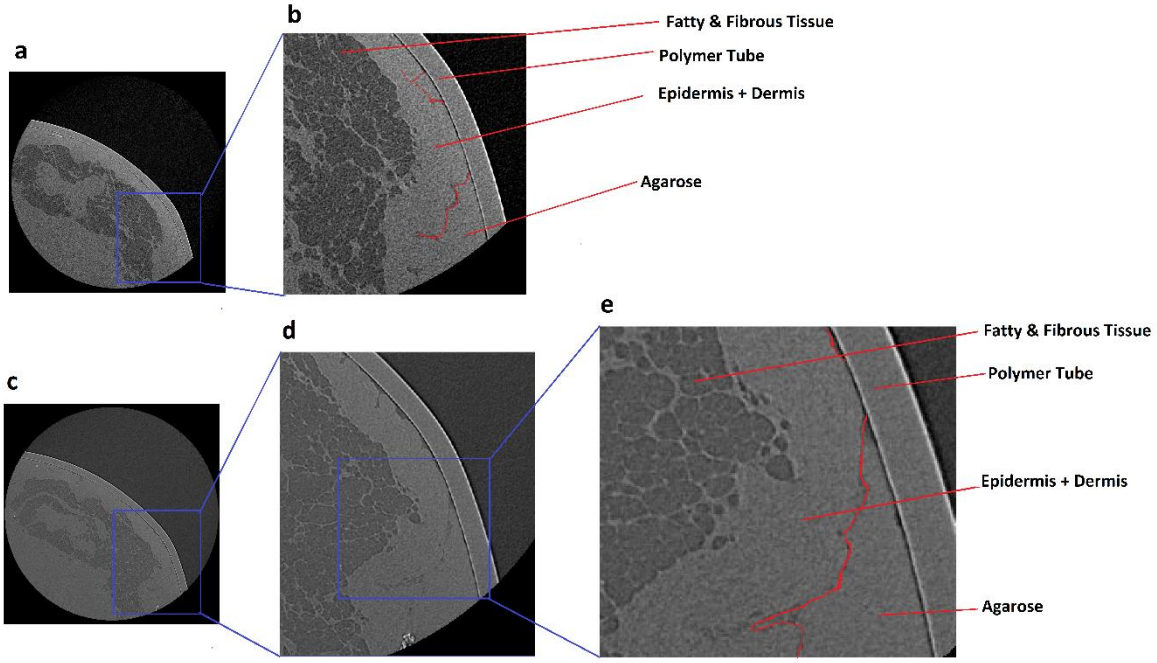

Figure 3. *Absorption (a, b) and phase contrast (c, d, e) images of chicken skin from top to down look were presented.*

Mouse skin sample which stabilized using agarose were investigated both absorption tomography imaging and phase contrast tomography imaging. The results belong to the mouse sample was presented in Figure 4. It was seen in Figure 4 that the images obtained both absorption tomography and phase contrast tomography was found more stable compared to the unsupported samples in Figure 2. In addition, finding images with higher image quality in Figure 4 indicates that using agarose as supporting material works. In absorption imaging in Figure 4 a and b, main parts like hair follicles, belong to mouse skin could be identified but details are still missing. For example, the epidermis and dermis could not be identified from each other. Moreover, the agarose solution used to support the skin covers around the skin. It is difficult to distinguish agarose support from mouse skin in the images (see Figure 4 a and b). At this point, Phase contrast imaging was found more reliable and has better contrast. Both the main parts and small details could be identified. Hair follicles, hairs, and the skin itself could be seen. Swirls and wrinkles of the skin could be seen. Epidermis and dermis could be identified and cuticled layers of the epidermis, which is called the cornified layer or horny cell layer, could be seen in detail. Dermis section was identified. The brightness of the agarose solution covering the skin was found quite similar to the dermis section. All in all, detailed sections of mouse skin could be visualized. After overcoming the stabilization and the achieving certain image quality problem, staining was applied to increase image quality.

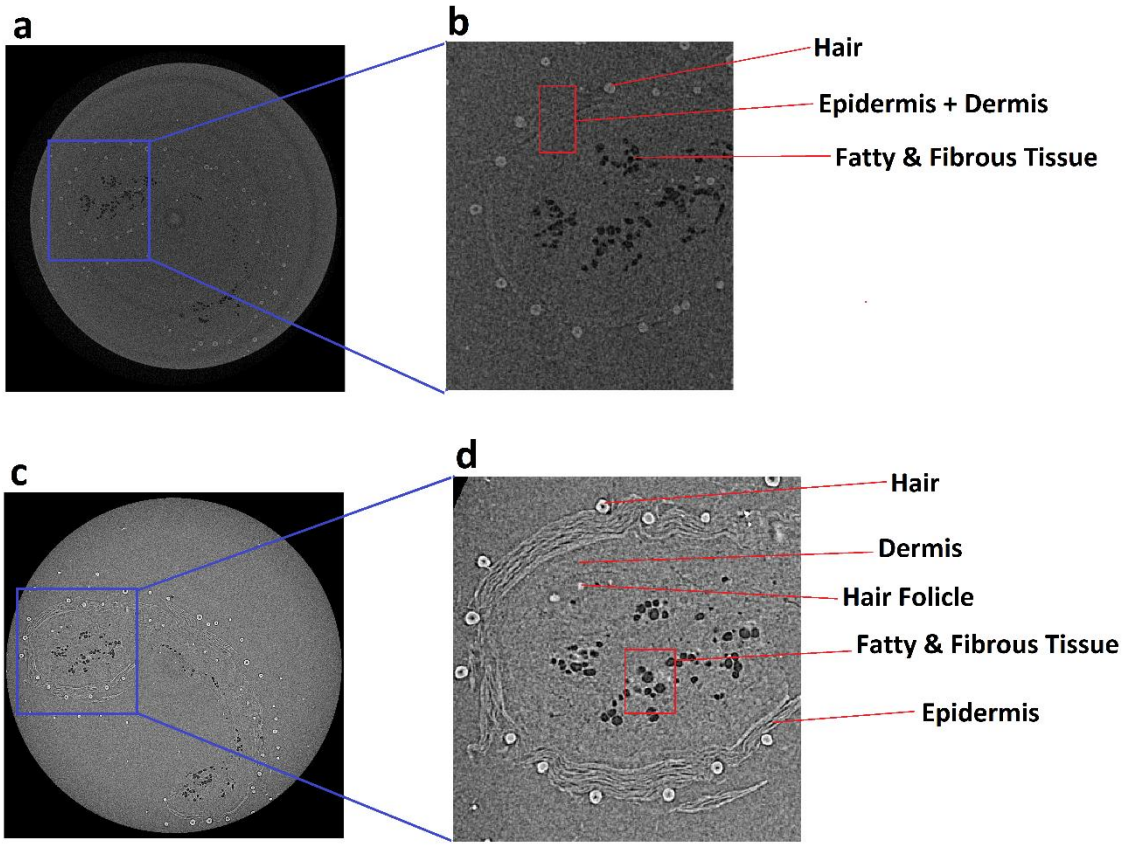

Figure 4. *Absorption (a, b) and phase contrast (c, d) images of mouse skin from top to down look were presented.*

B. STAINING

Staining is a common method used in tomographic application to increase the contrast of an inspected sample and to increase image quality obtained from tomography. In the previous section, the problems about the stability of sample and importance the sample support to obtaining certain image quality were discussed. In this section, the importance of staining methods and parameters to obtain skin images at certain contrast and high image quality will be discussed.

Since the structure of chicken skin was found fatty compared to the mouse skin, only mouse skin was investigated in this section. Mouse tail skin samples were stained using IKI solution. Three important results depending on staining were illustrated in the investigation of mouse skin in this section. Mouse skin stained with 0.5% IKI solution for overnight. Stained tissues than stabilized with 1% agarose in Eppendorf tube. The sample then scanned with absorption tomography and phase contrast tomography imaging.

Despite staining the skin with IKI, it was seen in the image that using Eppendorf tube instead of polymer tube decreases the image quality since the walls of Eppendorf tube is much thicker than polymer tube (around 1-2 mm) (See figure 4 a, b and Figure 5). It was seen that the obtained image quality in the Eppendorf tube was lower and less detailed. Main parts of mouse skin like hairs, hair

follicles, fibrous tissue, and intact epidermis and dermis could be identified but, details enabling us to distinguish the epidermis and dermis could not be seen.

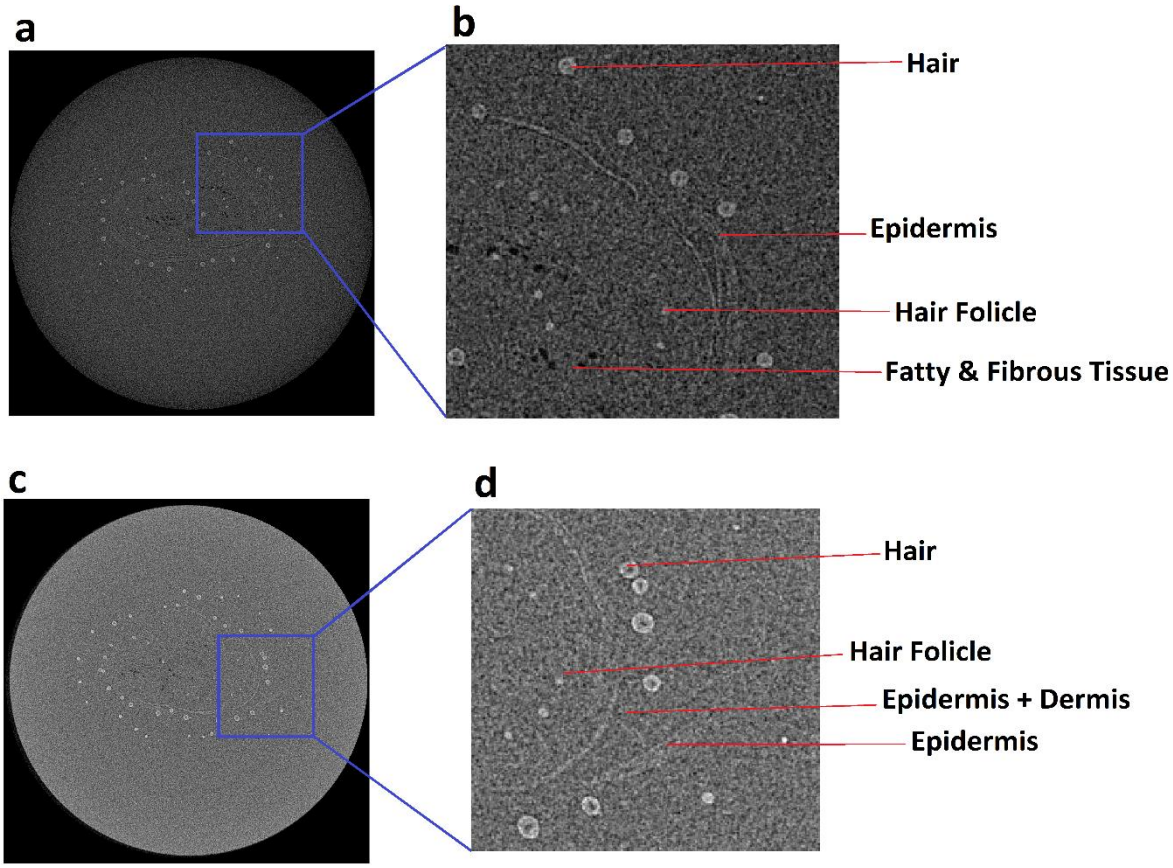

Figure 5. *Absorption imaging of mouse skin sample stained 0.5% IKI solution overnight.*

It was understood that both staining concentration and staining duration was found too low to increase image contrast and to obtain an image in high resolution. Therefore, staining concentration increased to 2.5 % and staining duration was increased to the two days. Figure 6 shows absorption imaging obtained from the mouse skin sample. Images pertaining to a look from left to right sectioning (Figure 6 a and b) and a look from top to bottom (Figure 6 c and d) were shown in the figure. It was seen that increasing staining time and staining duration make a positive effect on augmenting the image quality obtained from X-ray tomography scanning. Parts of the skin could be identified in detail and almost all parts of the skin could be observed. Layers of epidermis like cornified layer, granular cell layers, basal layers could be noticed with visual inspection. The separation between dermis and epidermis can be seen but not in perfect details. Hair follicles hairs, fatty and fibrous layers, and collagen structures could be identified. Unfortunately, clear separation between the skin tissue and agarose support could not be observed.

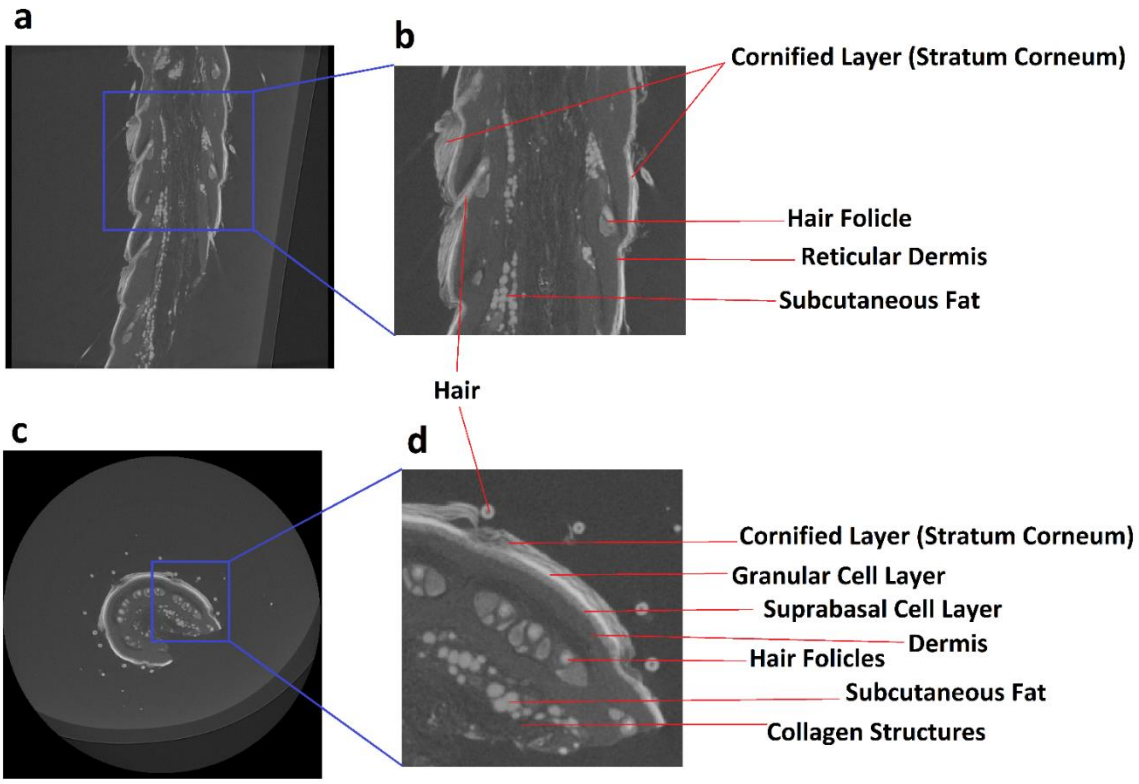

Figure 6. *Absorption imaging of mouse skin stained with 2,5% IKI for two days. Left to right view (a and b) and top to the bottom look (c and d) of skin placed in "C" shape were presented.*

Similar to absorption scan, high-quality images were also obtained from phase contrast scan as well. Figure 7 show phase contrast scan mages obtained from the mouse skin samples. Images belong to the left to right look (Figure 7 a and b) and top to the bottom look (Figure 7 c and d) were shown in the figure. The quality of images obtained from phase contrast gives great details about the structure of the skin. Almost all parts of the skin could be observed in the scan. A clear distinction between epidermis and dermis was observed. In addition, layers of epidermis could easily be identified. Fatty and fibrous structures, hair bulbs, hair follicles, and collagen structures were identified with visual inspection. No big quality difference between phase contrast and absorption scan was obtained.

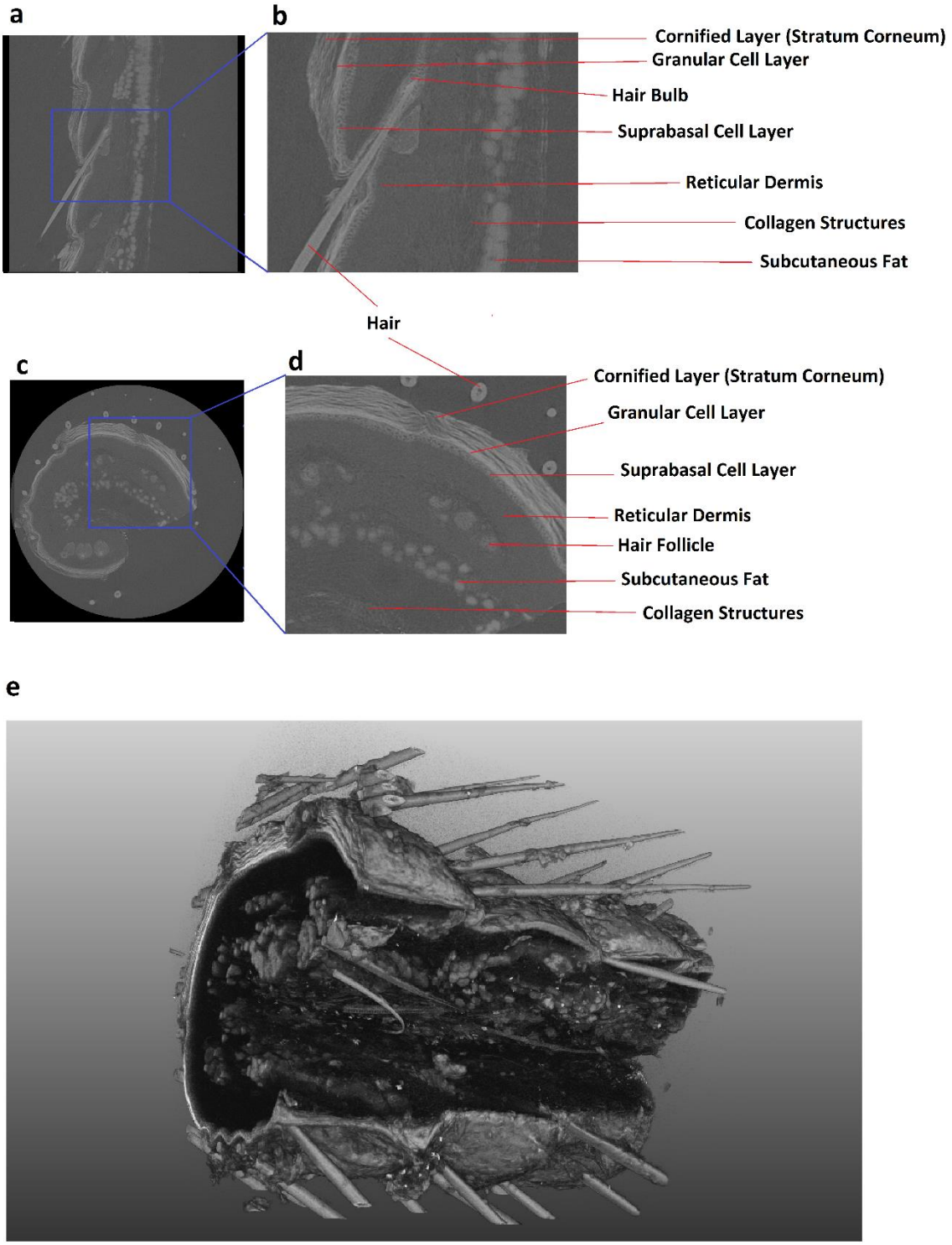

Figure 7. *Phase contrast imaging of mouse skin stained with 2,5% IKI for two days. Left to right view (a and b) and top to bottom look (c and d) of skin placed in "C" shape and 3D reconstructed image of the mouse skin (e)were presented.*

To assess the effect of staining duration, a new sample was prepared with increased staining duration. A mouse skin sample was stained with 2.5% IKI for 4 days, then put in Eppendorf tube and stabilized with 1% agarose Figure 8 shows absorption imaging obtained from the mouse skin sample. Images pertaining to a look from left to right sectioning (Figure 8 a and b) and a look from top to bottom

(Figure 8 c and d) were shown in the figure. The image quality was found high and details about the structure of the skin could be seen. The contrast in the outer parts of the skin is highlighted due to the long-term exposition of stain. The sample was found over stained. Both contrast and image quality were found good. All parts of the skin could be identified with visual inspection. Clear separation between epidermis and dermis could be seen and details like hair follicles, subcutaneous fat, cornified and granular cell layers, collagen structures could be identified. Also, clear separation between supporting agarose and skin could be seen in the figure.

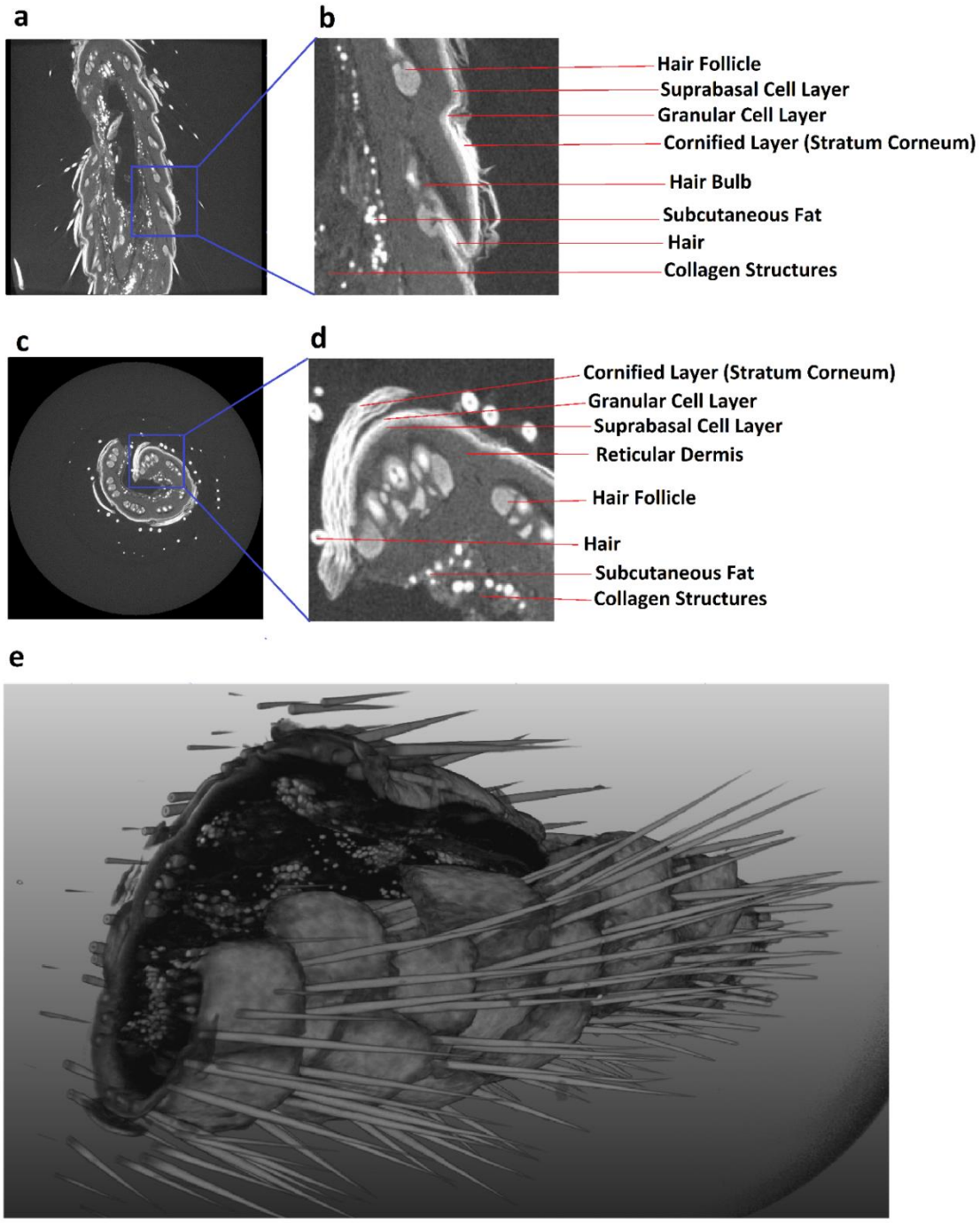

Figure 8. *Absorption imaging of mouse skin stained with 2,5% IKI for four days. Left to right view (a and b) and top to bottom look (c and d) of skin placed in "O" shape and 3D reconstructed image of the mouse skin (e) were presented.*

It was understood that staining concentration and staining duration carries great importance in achieving high quality tomography images. It was confirmed that the samples less stained did not give good contrast and it was even difficult to distinguish samples inspected sample from agarose support. Increasing stain concentration and staining duration for certain limits result in increasing contrast and image quality. Over exposition of the sample to the staining agents may result in over stained samples. Despite over staining, contrast obtained from the samples, image quality and details obtained from images were slightly lost. As proper staining conditions increase the image quality under and/or over staining may result in the decrease in the image quality and the contrast.

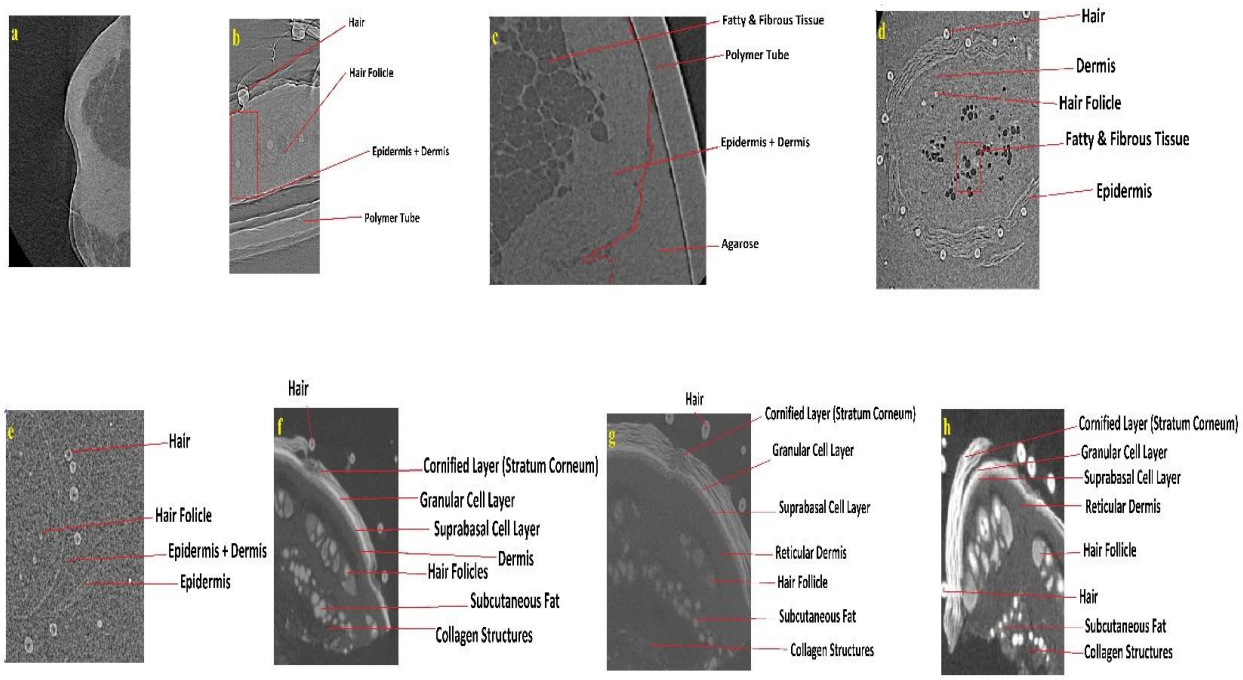

Figure 9. *Images show chicken skin without a support (a), mouse skin without a support (b), chicken skin with agarose support (c), mouse skin with agarose support (d), overnight 0.5% IKI stained mouse skin (e), two days 2.5% IKI stained mouse skin (f), two days 2.5% IKI stained mouse skin (g) and four days 2.5% IKI stained mouse skin (h), respectively.*

IV. CONCLUSION

X-ray tomography of soft tissues and soft structures especially in small sizes (in mm scale) always have been difficult due to certain problems affecting the quality of the images. Two major problems: stability of samples (*i*) and proper staining parameters (*ii*) directly affect the image quality. As far as the knowledge of the author, first time in the literature the skin was investigated using X-Ray microcomputed tomography. The effect of stability and staining protocols in image quality was illustrated. It was concluded that it was difficult to obtain a good image without stabilizing the sample. Good support increases the quality of images obtained from tomography. In addition, the container used in the measurement helps to prevent the sample from deterioration as well as support. It was also illustrated that applying staining protocols prior to the measurement significantly increases the image

quality obtained soft samples. Proper staining resulted in high-quality imaging; the detailed of structure of the skin could be identified with visual inspections of images. Almost all parts of the skin could be identified and becomes distinguishable after the application of proper staining protocols and clear separation of the parts can be observed. Under and/or over staining may results with a decrease in image quality. The contrast of image could be affected and ability to distinguish the parts of the skin may totally be affected. The results demonstrated the suitability of X-ray computed microtomography to visualize the skin and skin like soft tissues and to obtain the data of the skin in 3D. Proper stabilization and staining protocols applied to the sample prior to X-ray microtomography investigation, significantly increase the quality of obtained images in 3D. Obtaining high-quality images in 3D images helps many researchers to deeply understand the structures of soft tissues like skin. In additions, such images and information obtained from the X-ray tomography could be used in computer simulations, computational works, biomimetic materials development, and 3D and 4D printer implications for both medical and engineering purposes.

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