

IMAGE ANALYSIS OF APPLE TISSUE CELLS
AFTER MECHANICAL DEFORMATION*

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Abstract. Texture of apples depends on geometrical dimensions of the cellular mechanical skeleton. For quantitative measurements of the dimensions of cells an image analysis procedure should be developed which is proper to a certain imaging system. In this paper two different image analysis methods are applied: image segmentation and visual texture analysis (VTA) to images obtained by an optical microscope for which a special sample fixation procedure for apple tissue was developed. The image analysis was performed for thin sections of apple, cut by microtome from samples previously subjected to different strain levels: intact tissue, 5%, 10% and 15%. The experiment showed that segmentation of images from the optical microscope is possible after prior manual correction of the images of apple, while visual texture analysis (VTA) provides quantitative results for the size and shape of objects in the images automatically. The parameters obtained by the VTA correlate significantly with geometrical parameters obtained by the segmentation method. The highest correlation was obtained for product of the size of the horizontal and vertical linear structural element. Geometric dimensions of objects in the images increases with increase of the tissue strain level when observation plane is transversal to the direction of deformation. The effect can be affected by large amount of intercellular spaces in the apple, the volume of which decreases during deformation.

Key words: segmentation, visual texture analysis, apple

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INTRODUCTION

One of the most important factors affecting the quality of plant foods is texture. The textural properties of fruit and vegetables depend on many factors, among others: cell walls structure, cell walls thickness, cell walls chemical composition, pectin degradation level, turgor pressure within cells (Jackman and Stanley 1995), parenchyma cells arrangement (Zdunek and Umeda 2005 and 2006, Devaux *et al.* 2005), quantity and volume of intracellular spaces (Chanliaud *et al.* 2002) and permeability of cell walls (Pitt 1982, Pitt and Chen 1983).

Plant tissue is very susceptible to damage caused by different kinds of loading occurring during harvest, transport, processing and storage. Most of the injuries are irreversible and entail significant decrease of the quality of product (Van Zeebroeck *et al.* 2007). Bourne (2002) and Waldron *et al.* (2003) emphasise role identification of microstructural elements for apparent texture attributes of food. Texture properties of raw foods are strongly influenced by properties of the tissue mechanical skeleton (Zdunek and Umeda 2006). One of the properties of the mechanical skeleton which would influence on texture is cell size and orientation.

In order to describe the cellular structure of plant tissue different microscope techniques are usually used. Zdunek *et al.* (2004) applied confocal scanning laser microscope (CSLM) to obtain fluorescence images of potato and carrot parenchyma cell walls which were further automatically analysed quantitatively by segmentation. Guillemain *et al.* (2004) used confocal imaging for clustering cells according to their histology, also using segmentation. Semiautomatic segmentation of cell images from non-laser confocal microscope, i.e. tandem scanning reflected light microscope, was developed as well (Konstankiewicz *et al.* 2001). The general advantage of the confocal microscopy is distinct difference between cell walls and cell interior, which makes segmentation relatively easier. The second advantage is avoiding sample fixation. The CSLM was used for observation of changes of geometric dimensions of cells of potato and carrot tissue after deformation (Zdunek and Umeda 2006). It was shown that the relationship between deformation level and 2D (in the plane transversal to deformation applied) cell wall extension has third-order polynomial character. The disadvantage of the confocal microscopy is the relatively high price of the equipment and small observation area. Therefore, more effective techniques are developed as well. For example, the differences between the cellular structure of fresh and mealy apples were identified on the basis of images of fixated tissue from light microscope (De Smedt *et al.* 1998). Devaux *et al.* (2007) developed a macroscope technique and used grey level granulometry for quantification of tomato pericarp structure.

The grey level granulometry, often called visual texture analysis (VTA), is a promising tool for obtaining quantitative information about dimensions of objects within

image. The method was used for confocal images and calibrated using segmentation as the reference method (Zdunek *et al.* 2007). In the VTA method morphological operators like closing or opening are applied with increasing size of a structural element. Any type of structural element can be used. For object size analysis the square structural element can be used. For object orientation a vertical or a horizontal one would be useful. For each size of structural element a grey level of the image is measured. The change ratio of the grey level corresponds with the geometric dimensions of the objects. Details of the method are described by Devaux *et al.* (2007) and Zdunek *et al.* (2007).

In this paper two image analysis methods - the visual texture analysis and the segmentation method – are applied for microscope images obtained by means of a simple light microscope. In order to preserve the cellular structure for cutting thin sections by microtome, a sample fixation procedure for apple tissue was developed and is presented here. The procedure was applied for intact and deformed apple tissue. The goal of the research was to check the applicability of the visual texture analysis method for apple microstructure images from an optical microscope and to find a relationship between tissue strain level and changes of microstructure geometric dimensions.

MATERIAL AND METHODS

In the experiment apples (*Malus domestica* cv. Jumbo) were used that had been stored under cold-storage conditions at temperature of 4°C for ten months after harvest. Apples were delivered by the Integrated Production of Fruit “Stryjno – Sad” from Lublin province. Randomly selected 24 fruits of different size and shape were used in the experiment.

From each apple two cylindrical samples were cut from the equatorial region of the fruit, about 2 mm from the skin. The diameter of the samples was 12 mm and their height was 10.5 mm. The samples were subjected to uniaxial compression test. The mechanical tests were performed using a universal testing machine Lloyd LRX with 500 N load cell and the program Nexygen (Lloyd Instruments Ltd, Hampshire, UK) provided with the apparatus. Crosshead speed was 20 mm min⁻¹ which corresponded to 0.03 s⁻¹ strain rate for the sample initial height of 10.5 mm. The samples were compressed to the strain levels ε_i : 5%, 10% and 15% of the initial height of the sample. Next, the crosshead was stopped for 30 s for stress relaxation. From the test the maximum force F_m and the force relaxation F_r (the difference between the maximum force and the force after 30s) were analysed. For comparison, intact samples cut from each apple were analysed as well.

Sample preparation for microscopy

Immediately after the mechanical test, deformed samples and intact samples were trimmed to small rectangular pieces of 2 mm x 2 mm x 6 mm. Next, the pieces were immersed immediately in the first mild fixative solution (3% glutaraldehyde, 3% formaldehyde, 2.5% acetic acid and phosphate buffer up to 100 ml) for 4 hours and then in second, stronger, fixative solution (6% glutaraldehyde, 6% formaldehyde, 5% acetic acid and phosphate buffer up to 100 ml) for 18 hours. After the fixation, samples in fixative solution were placed in a desiccator under reduced pressure for 0.5 h in order to remove remaining air-bladders from the intracellular spaces in the tissue. Fixed samples were rinsed in phosphate buffer (pH 7.0) and then dehydrated in graded series of ethanol solutions (5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90 and 96%). Dehydrated apple samples were immersed in infiltration solution containing metacrylate resin with activator (Historesin, Leica, Germany) and 96% ethanol (1:1) for 0.5 hour under reduced pressure and then for 4 hours at 4°C. After infiltration the samples were embedded in embedding medium containing infiltration solution and hardener (Leica, Germany). Embedded samples were left at room temperature for 24 hours to harden. The samples were sectioned by means of rotary microtome Leica RM 2155 (Leica, Germany). Thickness of sections was 30 µm. Sections were mounted on slides and observed with a light microscope (lens E Plan 4x/0.01) equipped with a digital camera (Canon PowerShot A640). Colour images in JPG format with resolution of 800x600 pixels were taken. A pixel size was 2.5 µm. From each sample 8-9 slices were cut by microtome. From each slice one image was taken. Totally, for each deformation level about 50 images were obtained and analysed.

Image analysis

Images of apple microstructure were analysed in two ways: using the segmentation method and the visual texture analysis method. In order to segment images into objects representing 2D cells or intercellular spaces within the image manual correction was done firstly. From each object the interior was removed manually using CorelDraw8.0 software. After correction the images were analysed using a procedure prepared in Aphelion software (Aphelion, ADCIS, Hérouville Saint-Clair, France). The procedure uses watershed operator to label objects. For each object a set of geometric parameters like: area – S and perimeter – P , Feret diameters minimal F_{min} and maximal F_{max} , respectively, are automatically calculated and exported to a calculation chart. The results from the segmentation was treated as reference results for the visual texture analysis performed on the same images.

The visual texture analysis procedure was also prepared in Aphelion software basing on the method described by Devaux *et al.* (2007) and Zdunek *et al.* (2007). The procedure uses three structural elements: square, vertical linear and horizontal linear. The first step in the procedure is inversion of the images to negative. Next, the initial total grey level $G(0)$ is measured and next a closing morphological operator is applied with increasing size i of the structural element. At each step new total grey level $G(i)$ is measured and a relative difference $g(i)$ between resulting grey level and the source image grey level $G(0)$ is calculated using the formula:

$$g(i) = \frac{G(i) - G(i-1)}{G(0)}, \quad (1)$$

The maximum of the $g(i)$ function is determined. The size of the structural element at the maximum of $g(i)$ was named *SQR*, *VL* and *HL*, for the square, the vertical linear and the horizontal linear structural elements, respectively.

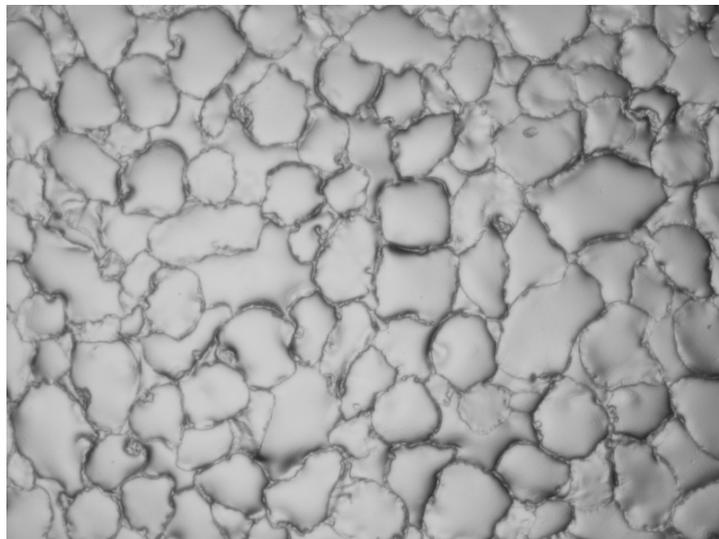
RESULTS AND DISCUSSION

Image properties and image analysis

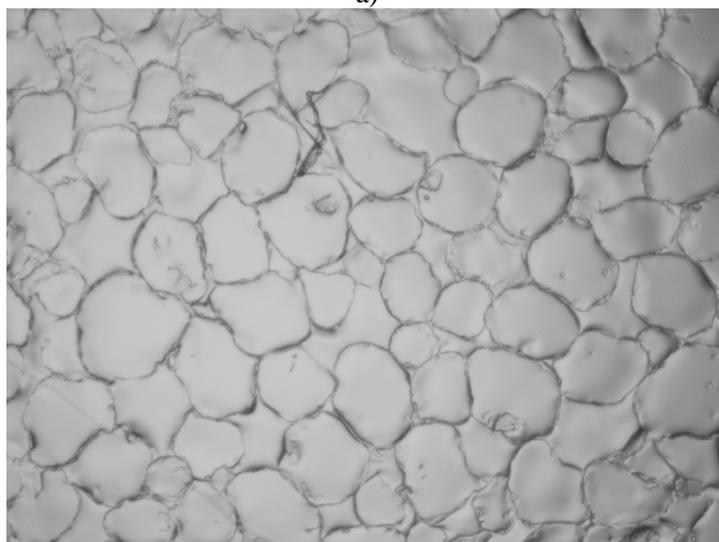
Examples of images of 30 μm sample sections obtained by the optical microscope after sample preparation are shown in Figure 1. In the images cells are clearly visible as convex objects. Concave objects are the intercellular spaces. In the further image analysis both types of objects were treated together in the present experiment. The property which is important from the point of view of image analysis is clear distinction between cell walls and cell interior. The cell walls have inhomogeneous grey levels in the sections, however in both image analysis procedures applied this is not important. Segmentation preceded by manual correction is insensitive to wall grey level if the wall is not broken. In the visual texture analysis the difference of grey level is normalized to the grey level of the initial image.

The images shown in Figure 1 present examples of intact apple tissue and the apple tissue after deformation at strain of 10% and 30 s stress relaxation (Fig. 1b). The compression axis is transversal to the images plane. After quantitative analysis of set of all images it was concluded that it is difficult to distinguish images of intact tissue and images after deformation. Therefore, image analysis is necessary to check changes of geometry of cells and intercellular spaces after deformation.

In the experiment 200 images were analysed in total. Applying the two image analysis methods (segmentation and visual texture analysis) allowed obtaining quantitative geometric parameters of objects within images.



a)

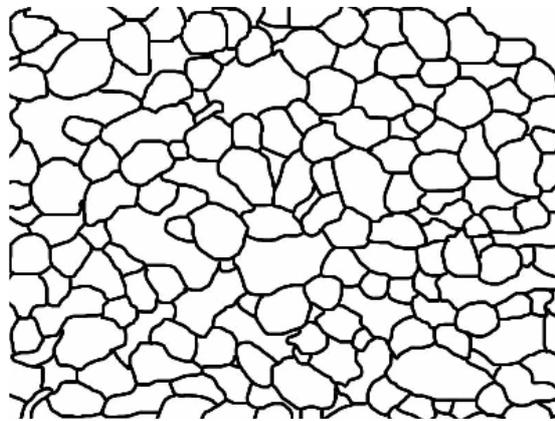


b)

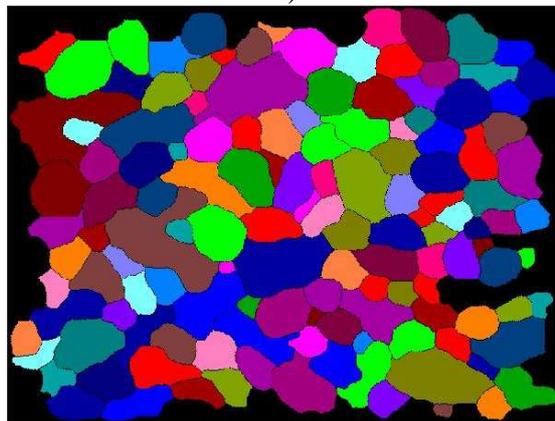
Fig. 1. Microscope images of intact apple tissue (a) and apple tissue after deformation (b – strain of 10% and 30 s stress relaxation)

The segmentation method would be treated as reference for the visual texture method. The segmentation provides accurate and direct result of objects geometric dimensions like its area - S , perimeter - P and Feret diameters F_{max} and F_{min} . Since the automatic segmentation for images from the optical microscope is difficult to per-

form, preceding manual correction was performed. The manual correction introduced some uncertainty of measurements of the object dimensions due to an operator decision. The operator chose line of the cell wall and removed pixels from the cell interior, which made the process subjective. Next, the procedure of segmentation, which uses watershed morphological operator, segmented the image into stitched objects automatically. The border of each object is in the middle of line that has remained after manual correction. The results of the segmentation are shown in Figure 2. As a result, the images are segmented into objects with relatively high precision compared to preliminary attempts at fully automatic segmentation where a lot of objects were not correctly reconstructed.



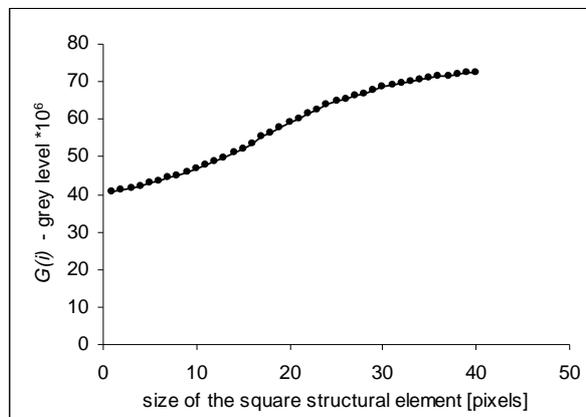
a)



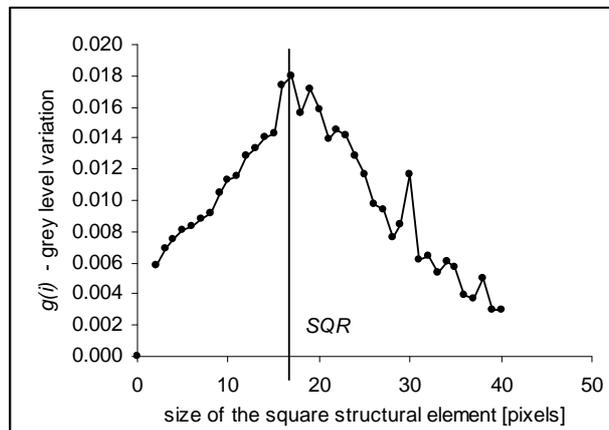
b)

Fig. 2. Example of manually corrected image of thin section of apple tissue obtained by the optical microscope (a) and its segmentation (b)

The visual texture analysis is much a simpler and fully automatic procedure. As it was shown by Zdunek *et al.* (2007), applying closing morphological operator with increasing size of the structural element causes increase of the image grey level. This is due to bolding cell walls of confocal images where cell wall has higher grey level than cell interior. In the case of thin tissue sections after sample preparation obtained by the optical microscope, the images should be inverted to negative first. After that, applying closing operator to the images also increases thickness of the cell walls. Increasing size of structural element causes continuous increase of the grey level $G(i)$ as shown as an example in Figure 3a.



a)



b)

Fig. 3. a) Increase of the image grey level $G(i)$ with increase of the size of the structural element, b) grey level variation $g(i)$ between resulting grey level and the source image grey level, $g(i)$ calculated using equation (1). SQR is the size of the square structural element at the maximum of $g(i)$

Zdunek *et al.* (2007) have shown that the grey level variation has third-order polynomial character. Two methods of finding the maximum of grey level variation would be used: 1) finding the size of the structural elements at second derivative equal zero or 2) using equation (1) and finding the size of the structural element at the maximum of $g(i)$ as it is shown in Figure 3b. The meaning of the parameter calculated by any method mentioned above is the size of the structural elements of closing operator when statistically a major part of objects is filled completely with brighter pixels. For 2bit images one can imagine as filling black objects completely by pixel value 255 (white). Therefore, this size of structural element at the maximum of grey level variation represents the objects size or one of its dimensions which is the most representative for the whole image.

Table 1. Correlation matrix for objects geometric parameters obtained by two image analysis methods: visual texture analysis and segmentation. All correlation coefficients are significant at $p < 0.01$

Para- meters	Visual texture analysis				Segmentation				
	SQR	HL	VL	$HL*VL$	S	P	F_{max}	F_{min}	$\frac{F_{max}^*}{F_{min}}$
SQR	1								
HL	0.457	1							
VL	0.332	0.292	1						
$HL*VL$	0.469	0.780	0.815	1					
S	0.463	0.382	0.484	0.555	1				
P	0.430	0.374	0.467	0.536	0.967	1			
F_{max}	0.421	0.355	0.457	0.519	0.944	0.984	1		
F_{min}	0.473	0.403	0.487	0.565	0.959	0.961	0.927	1	
$F_{max}^*F_{min}$	0.460	0.388	0.486	0.559	0.973	0.989	0.981	0.978	1

Using different shapes of structural elements provides different information about the size and orientation of objects within an image. The square structural element relates with the object area while the horizontal and the vertical linear elements relate with respective object dimensions in these directions or can provide information about objects orientation in an image. In order to confirm this hypothesis

and to find the calibration curve for images obtained by optical microscope the correlation coefficients between parameters obtained by segmentation and visual texture analysis methods were found (Tab. 1). It was found that in the case of images obtained by optical microscopy all relationships are linear between parameters analysed. It is different than for images of potato and carrot obtained by confocal microscope, when between the square structural element of visual texture analysis and 2D cell area from segmentation a square function was found (Zdunek *et al.* 2007).

Table 1 shows that significant linear correlation at $p < 0.01$ exists between geometric parameters obtained by these two methods. In general, taking into account that segmentation is the reference method, it can be concluded that the most accurate prediction of object geometric dimensions on images from optical microscope would be obtained using product of the size of the vertical and linear elements ($HL*VL$). This value has the same unit as area S and the product of $F_{max}*F_{min}$. It should be emphasized that visual texture analysis is a fully automatic method, therefore useful for analysis of a large amount of images from an optical microscope as it is usually required in quantitative measurements.

Changes of object geometric parameters after tissue deformation

Increasing deformation of apple tissue causes closely linear increase of force at the respective strain level (Tab. 2). The samples were kept for 30 s under constant strain due to force relaxation. During this process intracellular fluid is flowing out of the cells. This process is irreversible. As a result, the force relaxation increases with the strain level as well. From microstructure point of view, it causes that the cells do not fully return to their initial shape after crosshead removal. At the highest strain level used, 15%, the force relaxation is as much as 43% of its initial value and is significantly higher than for 5% and 10%. This is due to cellular structure fracturing at so high a strain for apple, as reported by Zdunek and Bednarczyk (2006).

Table 2 shows that all geometric parameters of objects in images of apple thin sections from optical microscope increase with increasing strain level. The smallest changes or no changes are observed at low deformation (5%). Further deformation causes continuous increase of the object size in the images.

As it is shown in model cell developed by Pitt (1982) and Pitt and Chen (1983), compression causes lateral extension of the cell due to incompressibility of the intracellular fluid. Assuming also that there is no fluid movement out of the cell, the cell volume remains constant during deformation. It causes that cell walls which are transversal to the force direction are stretched more than the walls longitudinal to the force. In this experiment, the observation plane was transversal to

the deformation direction, thus it was expected that the cells in this plane would increase in size, similarly as it was shown in the experimental observation by Zdunek and Umeda (2006) for potato and carrot using confocal laser microscope. The apparent effect depends on the strain rate, which means that the rate of the fluid flowing out the cells is crucial here. Apple tissue differs from potato and carrot tissue in much larger amount of intercellular spaces which is about 20%. The spaces are filled with gas and are easily compressible. During deformation, neighbouring cells can expand freely to the spaces and actually the volume of the spaces decreases. Therefore in 2D plane transversal to the deformation the size of the cells increases and the size of the intercellular spaces decreases for apple. Since in the image analysis applied in this experiment both cells and spaces are analysed as objects, the average effect of the object size increase after deformation for apple would be less significant than it can be for potato and carrot at similar strain rate. However, this should be confirmed in further experiment by direct comparison of images obtained in the same way for those materials.

Table 2. Mean values of the mechanical parameters and the object geometric parameters from the visual texture analysis and the segmentation method of images of thin section of apple tissue after preparation, obtained by optical microscope. Confidence intervals are at $\alpha=0.05$

Mechanical parameters		Visual texture analysis					Segmentation				
ϵ_t (%)	F_m (N)	F_r (%)	SQR (pix.)	HL (pix.)	VL (pix.)	HL^* VL (pix.)	S (μm^2)	P (μm)	F_{max} (μm)	F_{min} (μm)	$F_{max} * F_{min}$ (μm^2)
0	0	0	20	25	24	620	21216	774	196	145	28441
-	-	-	± 0.9	± 0.9	± 0.8	± 31	± 850	± 14	± 4	± 3	± 1070
5	17.1	27	19	24	24	581	21514	805	205	144	29727
-	± 1.36	± 0.01	± 0.8	± 1.1	± 1.1	± 39	± 880	± 16	± 5	± 3	± 1168
10	32.6	29	21	26	26	670	25310	843	217	154	33837
-	± 2.68	± 0.03	± 1.3	± 1.3	± 1.6	± 58	± 1779	± 28	± 7	± 5	± 2093
15	40.2	43	23	27	26	731	28328	887	228	163	37392
-	± 2.49	± 0.04	± 1.2	± 1.3	± 1.3	± 58	± 1651	± 24	± 6	± 5	± 1897

CONCLUSIONS

In the research, two image analysis methods for images from optical microscope were applied for apple tissue at different strain levels. The experiment has shown that:

1. Sample preparation (fixation) method adopted for apple allows cutting thin sections useful for simple optical microscope.
2. Segmentation of images from the optical microscope is possible after prior manual correction of the images of apple.
3. Visual texture analysis (VTA) provides quantitative results concerning the size and shape of objects in the images from the optical microscope. The parameters obtained by VTA correlate significantly with geometric parameters obtained by the segmentation method. The highest correlation was obtained for product of the size of the horizontal and vertical linear structural element. The advantage of the VTA is automatic and relatively accurate measurements.
4. Geometric dimensions of objects in the images increase with increase of the tissue strain level when observation plane is transversal to the direction of deformation. The effect can be affected by large amount of intercellular spaces in the apple which decrease volume during deformation.

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ANALIZA OBRAZU KOMÓREK TKANKI JABŁKA PO DEFORMACJI MECHANICZNEJ

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Streszczenie. Tekstura jabłek zależy od wymiarów geometrycznych szkieletu komórkowego. W celu ilościowych pomiarów wymiarów komórek konieczne jest opracowanie procedury analizy obrazów właściwej do użytego systemu jej obrazowania. W pracy zostały zastosowane dwie różne procedury analizy obrazu: segmentację obrazu oraz wizualną analizę tekstury VTA do analizy obrazów uzyskanych przy pomocy mikroskopu optycznego, dla którego opracowano specjalną procedurę preparatyki próbek. Analiza obrazu została zastosowana do cienkich skrawków tkanki jabłka ciętych przy pomocy mikrotomu z próbek poddanych różnym poziomom odkształcenia: tkanka nieodkształcona, 5%, 10% oraz 15%. Eksperyment pokazał, że segmentacja obrazu z mikroskopu optycznego jest możliwa po manualnej korekcie wstępnej obrazów tkanki jabłka, natomiast wizualna analiza tekstury (VTA) dostarcza informacji o wielkości i kształcie komórek w sposób automatyczny. Parametry uzyskane przy pomocy VTA korelują istotnie z geometrycznymi parametrami uzyskanymi w metodzie segmentacji. Najwyższe współczynniki korelacji uzyskano dla iloczynu poziomego i pionowego liniowego elementu strukturalnego. Geometryczne wymiary obiektów na obrazie wzrastają wraz ze wzrostem odkształcenia tkanki w płaszczyźnie prostopadłej do kierunku zadawanej deformacji. Efekt ten może być mniejszy w wyniku obecności znacznej zawartości przestrzeni międzykomórkowych, które zmniejszają swoją objętość podczas deformacji.

Słowa kluczowe: segmentacja, wizualna analiza tekstury, jabłko