

Effect of Cytochalasin B, Lantrunculin B, Colchicine, Cycloheximid, Dimethyl Sulfoxide and Ion Channel Inhibitors on Biospeckle Activity in Apple Tissue

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Abstract The biospeckle phenomenon is used for non-destructive monitoring the quality of fresh fruits and vegetables, but in the case of plant tissues there is a lack of experimentally confirmed information about the biological origin of the biospeckle activity (BA). As a main sources of BA, processes associated with the movement inside the cell, such as cytoplasmic streaming, organelle movement and intra- and extracellular transport mechanisms, are considered. The aim of this study is to investigate the effect of metabolism inhibitors, connected with intracellular movement such as cytochalasin B, lantrunculin B, colchicine, cycloheximid, dimethyl sulfoxide (DMSO) and mixture of ion channel inhibitors, injected into apples, on BA. Two methods of BA analysis based on cross-correlation coefficient and Laser Speckle Contrast Analysis (LASCA) were used. DMSO, lantrunculin B and mixture of ion channel inhibitors have a significant effect on BA, and approximately 74 % of BA of apple tissue is potentially caused by biological processes. Results indicate that the functioning of actin microfilaments and ion channels significantly affect BA.

Key words Apple tissue · Biological processes · Biospeckle · Image analysis · Intracellular movements · Metabolism inhibitors

Introduction

Biospeckle is a variable in time and space interference pattern, formed as the result of illumination of the living

organism by coherent light. Biospeckle is observed only for biological materials and is a result of the micro-movement of internal elements of a biological object. The measure of biospeckle pattern dynamics is biospeckle activity (BA), obtained by various methods of image analysis. At present, biospeckle is studied as a potential tool in medicine, plant biology and agriculture, for example, in real-time, non-invasive examination of microcirculation in different organs of the human body [1], non-destructive evaluation of viability of seeds [2], activity of parasites in living tissues [3], analysis of maturation and bruising of fruits and vegetables [4] and monitoring of apple shelf life [5].

The physics of biospeckle is well-developed [6], but experimentally confirmed information about the biological background of biospeckle of plant tissues are scarce. BA is the result of movements inside a tissue. Three types of processes can, therefore, be taken into account as its source: physical processes (diffusion, Brownian motion, evaporation from the surface of biological object), instability of measuring equipment and biological processes such as: cytoplasmic streaming, organelle movement, transport of substances in the cell, transport of water and solutes in vascular bundles, cell growth and division, etc. [7]. However, there is a lack of experiments explaining which of these processes is the main source of BA.

The goal of this study is to investigate the effects of cytochalasin B, lantrunculin B, colchicine, cycloheximid, dimethyl sulfoxide (DMSO) and a mixture of ion channel inhibitors (ICI) (anthracene-9-carboxylic acid (A9C), tetraethylammonium chloride (TEA), gadolinium chloride and diethylstilbestrol (DES)) on BA. Except DMSO, these substances non-destructively and highly selectively influence processes associated with the movement of cytoplasmic organelles and substances.

Cytochalasin B inhibits polymerization [8] and lantrunculin B causes depolymerization [9] of actin filaments, and both,

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cease the transport associated with the actin cytoskeleton [10] as well as cytoplasmic streaming [11]. Colchicine, binding to tubulin dimers, prevents microtubule polymerization, stopping their reorganization [12]. In turn, cycloheximide - an inhibitor of translation - blocks protein synthesis and can potentially reduce the number of emerging and moving scattering centres in the cytoplasm in the form of the protein [13]. Since changes in intracellular ions concentration alter cytoplasmic streaming [14], an ICI, blocks the transport of K^+ , Ca^{2+} , anions and protons [15], and can affect movements of cytoplasm and a number of secondary processes.

Materials and Methods

Experiments were conducted on apples (*Malus domestica* Borkh cv. 'Idared') received from The Institute of Horticulture (Skierniewice, Poland). Fruit were harvested in the optimum harvest window based on the ethylene and starch tests, and stored in a cold room at 2 °C in a normal atmosphere for 4 months. About 24 h before experiment apples were conditioned at room temperature.

In the experiment, nine different solutions at amounts of 100–200 μ l were injected into apple tissues with a 1 ml syringe (Fig. 1):

- SS (Standard Solution) - containing deionized water, 1 mM KCl, 1 mM $CaCl_2$ and 50 mM mannitol (pH 7 – TRIS/MES) - as a control for the injection method and the base for preparation of solutions of active substances.
- 1% DMSO (1 % solution of dimethyl sulfoxide in SS, v/v) - as a control of the effect of 1%DMSO (used to solve cytochalasin B, lantrunculin B, A9C and DES) on BA.
- MANN (0.5 M solution of mannitol in SS) - as a control of effect of hypertonic solution on BA.
- CB (0.5 mM solution of cytochalasin B in 1%DMSO) to verify the effect of actin microfilaments' polymerization inhibition on BA.
- LANTR (0.5 mM solution of lantrunculin B in 1%DMSO) - to verify the effect of actin microfilaments' depolymerization on BA.

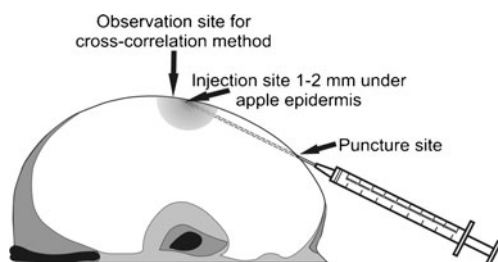


Fig. 1 Schematic presentation of the injection method

- COLCH (2.5 mM solution of colchicine in SS) to verify the effect of microtubules' reorganization inhibition on BA.
- CYCL (1 mM solution of cycloheximid in SS) to verify the effect of the inhibition of protein synthesis on BA.
- ICI (mixture of ion channel inhibitors in 1%DMSO in 1 mM concentration each: A9C, TEA, DES, gadolinium chloride) to verify the effect of the inhibition of ion transport through the cell membranes on BA.
- 100%DMSO - to verify the effect of cell destruction on BA.

Biospeckle Activity Measurements

BA was evaluated by two different systems and approaches. The first method based on cross-correlation coefficient was used to precisely quantify the BA on a small area of apple surface, near to the place of injection. The second method, Laser Speckle Contrast Analysis (LASCA), was used for qualitative evaluation of BA through the creation of maps of the BA of the whole visible side of the fruit.

The system for cross-correlation BA measurements [5, 16, 17] (Fig. 2) consists of a diode laser (30 mW, $\lambda=635$ nm, Newport, USA), with a 20 \times laser beam expander (Edmund Optics, USA) to illuminate the sample, and a CCD camera (Monochrome FireWire Astronomy Camera DMK 21AF04.AS, The Imaging Source Europe GmbH, Germany) with a 25 mm objective and a 20 mm extension ring to detect scattered light. The distance between camera and object was 37 mm and that between laser and object was 180 mm. The illumination angle was 30°. Experiment was conducted in a dark room, and the light source in addition to low-intensity laser light was a computer. Expanded laser light intensity registered at distances corresponding to the laser-object in both systems was less than 1 μ mol $m^{-2} s^{-1}$. Biospeckle activity was recorded as uncompressed movies (AVI, 8 bits, RGB24 codec) lasting 4 s

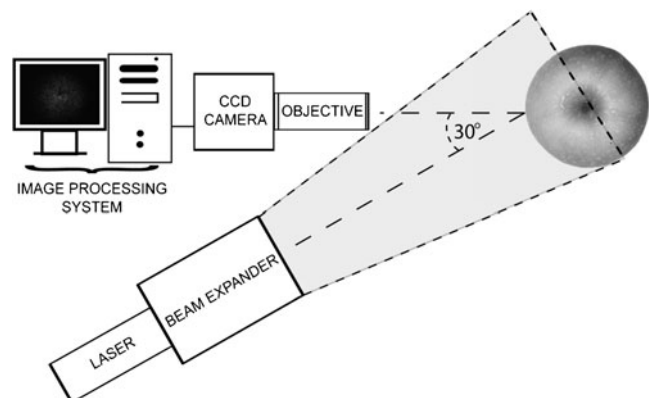


Fig. 2 Scheme of experimental setup for BA measurements (top view)

with 15 frames per second. The image resolution was 320×240 pixels, which corresponded to an observation area of 7.5 mm². The image exposure time was 1/250 s. Gain and brightness was found experimentally so as to avoid overexposures of pixels on the image histogram. The image acquisition settings were kept unchanged during the experiment.

BA was determined by means of the “corrcoef” function available in Matlab as a cross-correlation coefficient (C⁴) between speckle frames recorded with 4 s lag. Then, BA was calculated as 1-C⁴.

The second system for biospeckle measurements using the LASCA method consisted of the same elements (Fig. 2), and the same settings were used as in the cross-correlation method, with the exception that the camera lens had a 5 mm extension ring, distances from camera to object and from laser to object were 450 mm 630 mm, respectively, and a 400× laser beam expander was used. This allowed observation an area of about 70 cm², which was enough to capture the whole side of the apple in a single frame.

LASCA is a technique using the modified Briers contrast method, which allows showing the spatial distribution of speckle activity [18]. The LASCA algorithm is based on calculations of spatially or time-averaged contrast over the local square window of pixels. In this study, the contrast value C_{x,y} was calculated for each pixel separately, according to equation:

$$C_{x,y} = \frac{\sigma_{x,y}}{I_{x,y}} \tag{1}$$

Where $\sigma_{x,y}$ and $I_{x,y}$ are the standard deviation and the mean intensity, respectively. Both values were calculated for pixels in location x, y , in 60 frames. A threshold value of mean intensity equal to 5 was used, in order to reduce the influence of camera noise in less illuminated areas of the image. The resulting contrast map was spatially averaged using a 3×3 pixel size overlapping the square window. This operation was carried out in order to smooth generated images of BA. High contrast values on images obtained using this technique occur in areas with high speckle fluctuations. In areas where there are no fluctuations, the spatial contrast is reduced.

Experimental Procedure

To determine the effect of solutions of chemicals on BA, 12–24 apples for each solution were tested (Table 1), using both, cross-correlation and LASCA method.

Apples were placed in a measurement setup and recording of the movies began. After 60 min of recording, apples were removed from the setup, appropriate solution under the apple skin was injected (what took about 30 s), and apples were placed in the setup again for another 600 min. In the cross-

Table 1 Characteristics of BA before and after the injection of solutions of metabolism inhibitors

	SS	1 % DMSO	MANN	CB	LANTR	COLCH	CYCL	ICI	100 % DMSO
MBA±SD					0.61±0.002 ^a (n=156)				
BA during last 300 min. of experiment±SD	0.626±0.021 ^a (n=12)	0.637±0.043 ^a (n=15)	0.607±0.042 ^a (n=13)	0.540±0.056 ^a (n=24)	0.492±0.103 ^b (n=20)	0.634±0.034 ^a (n=15)	0.722±0.081 ^a (n=19)	0.370±0.137 ^c (n=13)	0.163±0.064 ^d (n=24)
Change of BA (%)	+1.5	+3.24	-1.62	-12.47	-20.25	+2.75	+17.01	-40.03	-73.58
Percentage effect of individual solutions on the BA resulting only from biological processes (in relation to BA of 100% DMSO) (%)	2.04	4.4	2.2	16.96	27.53	3.74	23.12	54.40	100

The same superscript letter means no significant difference at $p=0.05$. + or - in the row “Change of BA (%)” is an increase or decrease of BA after injection, respectively
MBA mean biospeckle activity before injection, SS standard solution, 1% DMSO 1% solution of dimethyl sulfoxide, MANN solution of mannitol, CB solution of cytochalasin B, LANTR solution of lantrunculin B, COLCH solution of colchicine, CYCL solution of cycloheximid, ICI solution of mixture of ion channel inhibitors, 100%DMSO 100% dimethyl sulfoxide, n number of apples examined

correlation method for each individual fruit, during 660 min a stack of 330 movies lasting 4 s each, with an interval of 2 min, were recorded using custom made software. For each apple, whole recording time was divided in three parts for BA calculations. The first 60 min were considered as the reference for the condition of apples before injection. The second part, the next 300 min, consisted of the injection of solutions and the stabilization of biological processes after injection. The third part, lasting 300 min, was the monitoring of effects which occurred as a result of the injected solutions (Fig. 3). The observation area was set to about 2–3 mm over the place where the solution was injected (Fig. 1). After measurements for the cross-correlation method, one 4 s movie for the LASCA method was recorded. After experimentation, tissue samples of 150 μm thickness taken exactly from the injection place were cut by VT 1000S Leica vibratome and imaged with a SZX16 Olympus stereoscope in dark field mode.

Statistical Analysis

Mean values of BA with standard deviations and other statistics were calculated using STASTICA[®] 8.0 (StatSoft, Inc. 1984–2008). One-way ANOVA with Tukey HSD post-hoc was used for checking the effect of injected chemicals on BA. The significance of effects was determined at $p < 0.05$.

Results

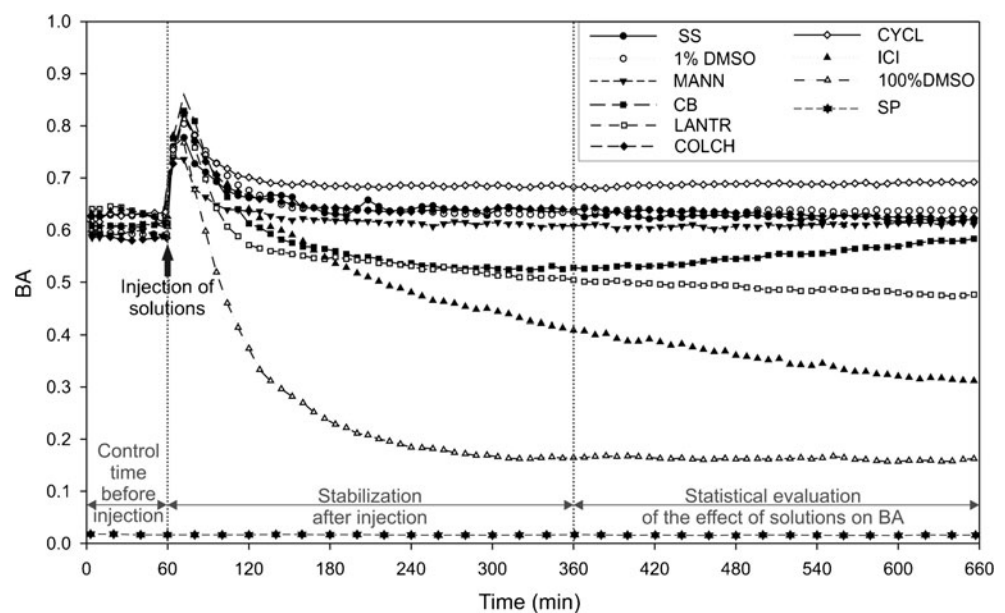
Before injection of solutions (Fig. 3, 0–60 min of the experiment), in all cases BA was stable. Mean biospeckle activity (MBA; approximately 0.617) was calculated for all apples

before injection and used as a reference for the estimates presented below. BA of apples before injection did not significantly differ.

Just after injection of solutions, indicated in Fig. 3, the BA suddenly increased and its stabilization lasted for at least 60 min. The solution effects were statistically analysed based on the data from the last 300 min of measurements (Fig. 3, 360–660 min of the experiment), because within the first few hours after injection BA could be affected by stress relaxation in the tissue. In Table 1, averaged values of BA for each solution during the last 300 min of the experiment are shown. In the case of SS, 1 % DMSO, MANN and COLCH, BA stabilized after injection at a value equal or slightly higher than before injection (Fig. 3). **Statistical analysis** showed no significant effects of these solutions on BA in comparison to apples before injection. A statistically significant effect was found ($F=45.816$, $p < 0.000$) after application of 100 % DMSO, ICI and LANTR. A transient but significant effect was observed after CB application (Fig. 3, between 240 and 360 min of the experiment). The strongest effect was observed after application of 100 % DMSO which caused, during the last 300 min of the experiment, BA to decrease by about 74 % of MBA. For ICI and LANTR, BA decreased by about 40 % and 20 % of MBA, respectively (Table 1).

Examples of apples appearance at about 600 min after injection, biospeckle images, results of LASCA and micrographs of injection place, for SS and solutions for which a significant effect was obtained (LANTR, ICI, 100 % DMSO) are presented in Fig. 4. Apples after injection of solutions, which are not presented in the Fig. 4 are similar to SS. Places of injection are marked by an arrow and black point in row A. One can observe visible bruising around the injection place on the skin of apples (Fig. 4, row A) which appeared

Fig. 3 Biospeckle activity before and after injection of solutions indicated in the legend. Additionally, SP is BA observed on a steel plate



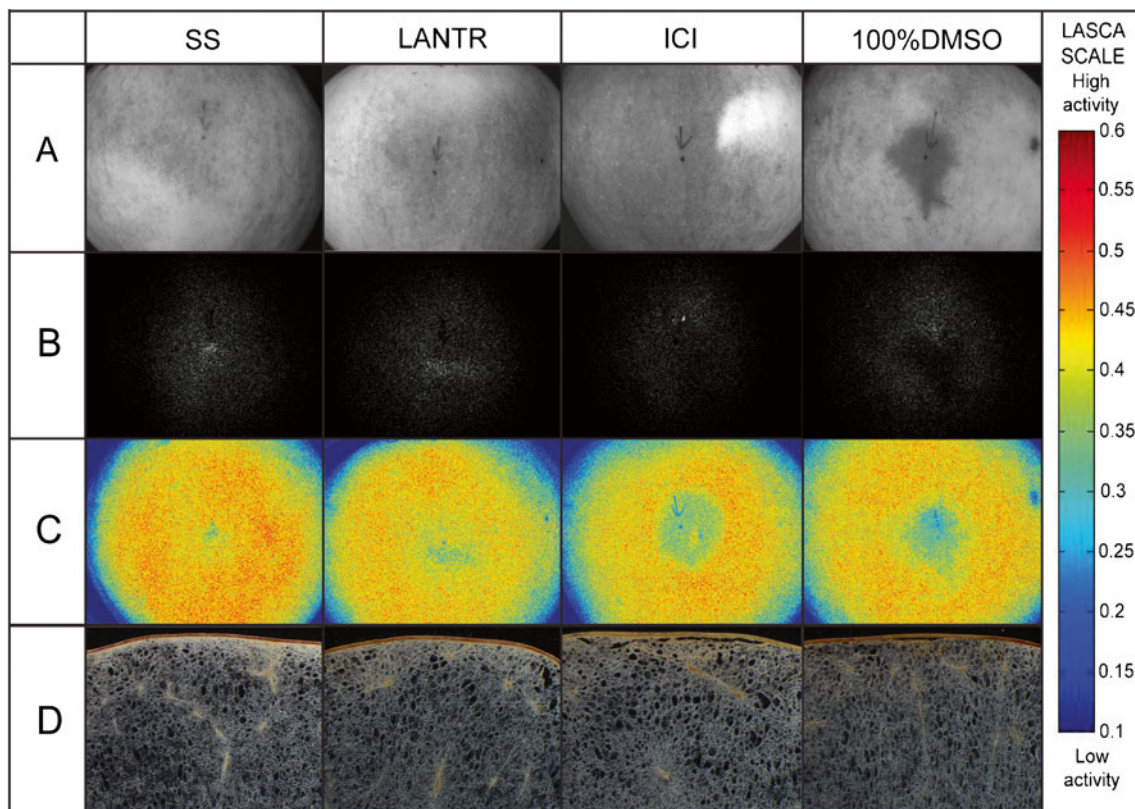


Fig. 4 Examples of: **A** apples 600 min after injection and just before recording a biospeckle patterns for LASCA, **B** biospeckle pattern recorded for LASCA analysis, **C** results of LASCA analysis, **D** micrographs of apple tissues close to the place of injection. Scale for LASCA

analysis is presented beside (values on the scale are not equal with BA derived from the cross-correlation method). Width of images shown in the rows **A-C** is 5 cm and in **d** 8 mm

only for 100 % DMSO. LASCA analysis (Fig. 4, row C) showed that areas with low BA near the place of injection, occurred in the case of 100 % DMSO, LANTR and ICI - which is consistent with the results of cross-correlation analysis. Micrographs (Fig. 4, row D) revealed that in the case of 100 % DMSO and ICI, in the place of injection, a free space existed between apple epidermis and parenchymatic cells. It was noticed that during the injection of chemicals, in 60–70 % of cases, blisters appeared in response to fluid pressure, also in the case of SS and LANTR. It was noted that blistering depended on applied pressure and on the properties of fruit tissue. For 30–40 % of apples where the blisters did not appear, injected fluid spread easily in the tissue under the fruit skin, and that was clearly visible on the skin. Additionally, these apples did not differ in terms of BA or appearance a few hours after injection.

Discussion

Injection or infusion of a solution of chemical substances into the stems of vascular plants is used in plant physiology in situations, when a systemic effect and relatively low tissue damage is needed. This method (Fig. 1) was adapted for the

application of chemical factors into apple tissue, to reveal a BA response to changes of selected biological processes.

Puncturing of fruit epidermis and parenchymatic cells, and application of the fluid under pressure, affected the structure of the tissue in areas where injected fluid reached. This resulted in the formation of blisters and free space under the apple skin (Fig. 4, row D). Injected solutions did not propagate systemically, but remained within a few centimetres of the injection site. However, no browning occurred at the treatment place after application of SS, LANTR, ICI (Fig. 4) and 1 % DMSO, MANN, CB, COLCH, CYCL (not shown), even after prolonged storage following the experiment.

BA before and after injection of control solutions (SS, 1 % DMSO and MANN) was similar (Table 1). It should be noted that just after injection BA suddenly increased, probably due to stress relaxation in the tissue after pressure treatment and the propagation of the injected fluid, but in the case of SS, 1 % DMSO and MANN, BA stabilized over the next 1 h at a value equal or slightly higher (difference not significant) than before injection. Lack of browning and unchanged BA a few hours after injection of SS, 1 % DMSO and MANN suggests, that the application of hydrostatic pressure only caused cell-to-cell debonding, without disruption of cell

membranes and the maintenance of cell function. The above observations of tissue appearance and BA before and after injection of control solutions suggest, that the method of injection has no significant effect on the presented results, despite some damages to the structure of tissues.

The results of cross-correlation analysis (Table 1) showed, that the disruption of cell membranes by 100 % DMSO, and the rapid death of cells at the application place, caused a decrease of BA by about 74 % in relation to MBA. Therefore, the BA observed during the last 300 min of the experiment was probably the result mainly physical phenomena and hardware noises, and can be treated as the lowest BA caused by the functioning of living cells. The remaining 26 % of the BA is the result of hardware noises and physical processes. BA measured on a steel plate (SP) could be used to distinguish BA from noise (BA for SP=0.015; Fig. 3). The difference between BA obtained after application of 100 % DMSO and SP, is the result only of physical processes inside the tissue and amounted to 24 %. According to this calculation BA caused by hardware noises is 2 %. Moreover, assuming that 100 % DMSO destroys all living cells near the area of injection, the percentage effect of other substances on the MBA resulting only from biological processes could be calculated (Table 1). If the 74 % decrease in total BA after injection of 100 % DMSO, corresponds to a 100 % stop of biological processes in apple tissue, ICI and LANTR, for which a statistically significant effect was obtained, decreased BA in relation to the 100 % DMSO by 54 % and 27 %, respectively. These two percentages reflect contributions of ICI and LANTR to all biological processes influencing BA.

ICI caused the second major effect on BA (decrease of BA by about 40 % compared to MBA and a 54 % share in BA resulting only from biological processes). The composition of ICI was chosen to inhibit the movement of the major inorganic ions through the cell membrane. The ion fluxes probably did not have a direct impact on the BA, because the size of the ions was too small relative to the wavelength of the laser used. However, multidirectional secondary effects could directly affect the movement of larger organic molecules and organelles, which are considered to be the source biospeckle activity. The combined effect of a reduction in the transport of organic molecules coupled with the transport of ions, the inhibition of conformational changes in carrier proteins, the lack of proper regulation of osmotic pressure in the cell and disruption of cell signalling caused a decrease in BA. The functioning of ion channels is also directly connected with cytoplasmic streaming [19]. During action potentials in *Chara* and *Nitella* cells, cytoplasmic streaming stops, which is probably connected with an increase in the concentration of Ca^{2+} in the first phase of action potential [20] and propagation of information about stimulus by calcium signalling [21], or with the direct effect of divalent ions (especially Mg^{+2}) on the actin cytoskeleton [22]. More

information on the relation between ion transport and BA, can be obtained in an experiment using single ion channel inhibitors.

Considering contribution of cytoskeleton to the movement in the cell and consequently to apparent BA, the effects of CB, LANTR and COLCH should be included. It was assumed that both, actin filaments and microtubules, are involved in the generation of cellular movement, and the inhibition of main processes connected with these structures (e.g.: reconstruction of the cytoskeleton, cytoplasmic streaming, transport substances or organelle positioning) will reduce BA. However, the results of experiments showed, that only substances connected with inhibition of actin filaments (CB, LANTR) decreased BA, while in the case of COLCH, BA only slightly increased. In the case of COLCH it should be taken into account, that the lack of effect can also be the result of colchicine's low affinity to apple tubulin [23] or low variability microtubules in apple cells. In conclusion, only the inhibition of functioning of the actin part of cytoskeleton affected movement inside the cells, which resulted in a significant decrease of BA (decrease of BA after injection of LANTR by about 20 % compared to MBA and a 27 % share in BA resulting only from biological processes).

Inhibition of protein synthesis at ribosomal stage by CYCL was expected to decrease BA due to a decrease in the number of scattering centres in the cytoplasm in the form of proteins and protein transport vesicles. However, the results of this experiment indicated the opposite, an increase tendency of BA was noted by about 17 % (not significant). This may be caused by starting additional or an increase in the existing rate of cellular mechanisms or by an increase in the amino acid content [24], which also could speed up biospeckle formation.

Information obtained by cross-correlation analysis are confirmed by LASCA (Fig. 4), which allows for spatial analysis of BA. In the case of 100 % DMSO, ICI and LANTR, around the area of injection, a clear decrease of BA was visible, while for other solutions the areas with decreased activity were not present.

In the light of the information presented above, it is possible to discuss practical application of the biospeckle phenomenon in fields of food science and biology. Cellular processes as cytoskeleton reconstruction, cytoplasmic streaming and ionic fluxes between cellular compartments and through the cell membrane, that have the greatest impact on BA, are changeable during plant development, maturation and aging as well as may be disrupted as a result of mechanical damage or pathogen attack. At present stage of advancement of methods of biospeckle image analysis, biospeckle techniques could be applied to determination the general rate of metabolism during the fruits life and for creation of spatial maps of BA of the surface of the whole fruits, which may be useful for early detection and visualization of mechanical

damages and infections. Sensitivity of biospeckle to vibration does not allow the use on production lines, but potentially can be used in long-term monitoring of the metabolic state and health of the agricultural products, for example during shelf life or cold storage.

In addition to vibration, use of biospeckle techniques is currently also limited due to inability to distinguish the dynamics generated by specific metabolic or physical process in the overall biospeckle signal. The solution of these problems may be the development of new measurement methods based on frequency analysis, which can open new possibilities for the study of specific physiological processes in the green parts of plants. An example of potential application of biospeckle in this case, could be the study of fruits or leaves physiology, where, non-destructively, cell wall and cytoskeleton reconstruction, cytoplasmic streaming or water status during development and aging could be measurable. Thus, at present, applications of biospeckle for fruits quality evaluation are possible, but wider application in fields of food technology and plant physiology require more specific models and theoretical studies.

Conclusions

In summary, this study presents the evidence that approximately 74 % of BA observed in apple tissue is potentially caused by the life processes. Action of the actin part of cytoskeleton and processes connected with functioning of ion channels and proton pumps make a significant contribution to the total BA. Obtained information indicates that biospeckle, as a simple and non-destructive technique, can be developed toward monitoring not only fresh produce quality but also specific biological phenomena like cytoplasmic streaming or intercellular transport.

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