Warsaw Uniwersity of Life Sciences
Faculty of Veterinary Medicine
Department of Physiological Sciences

prof. dr hab. Tomasz Motyl

Department Physiological Sciences:

- Division of Biochemistry
- Division of Dietetics
- Division of Physiology

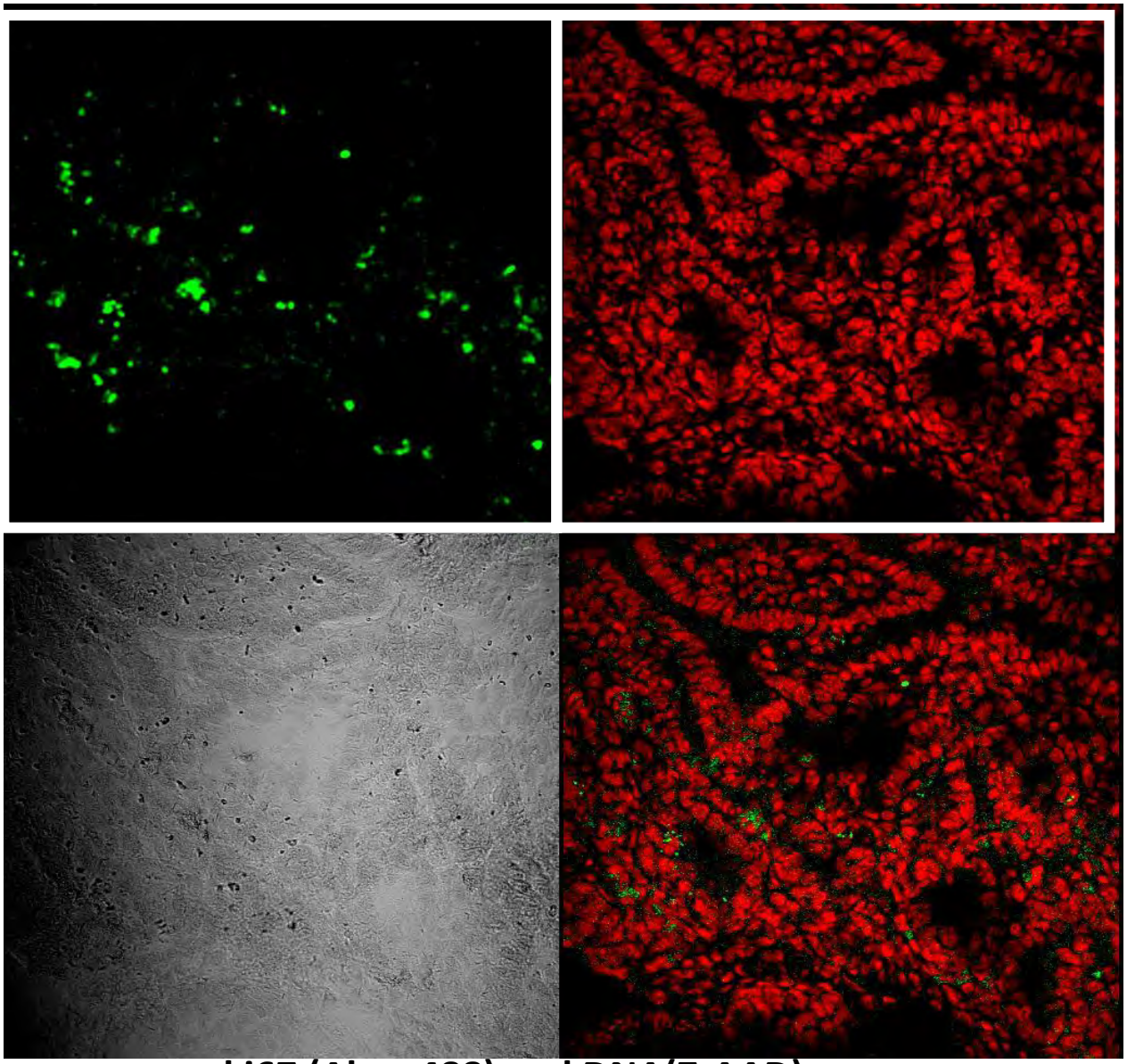
29 employees:

- 17 teachers
  - 8 titular professors
  - 3 associated professors
  - 6 assistant professors
- 12 technician (in it 3 with the PhD)
- 21 PhD students

a)
b)
c)
d)

„ in tissue cytometry”

- MicroImage,



ki67 (Alexa488) and DNA(7-AAD)

A CYTOMETRY APPROACH TO STUDY THE REBUILDING OF INTESTINAL MUCOSA IN WEANED PIGS FED WITH DIETARY NUCLEOTIDES

Joanna Bierła[1], Michał M. Godlewski[1,2], Artur Strzałkowski[1], Daniel Martinez-Puig[3], Beata Pająk[4], Anna Kotunia[5], Carles Chetrit[3], Romuald Zabielski[1]

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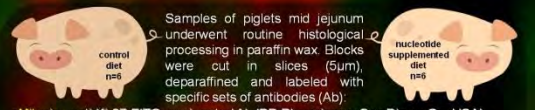
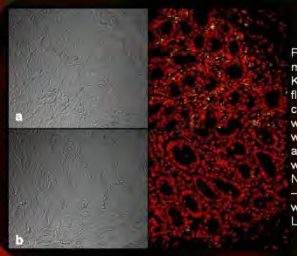
In the weaning period, transition from sow's milk to the post-weaning diet causes the withdrawal of important nutrients as milk nucleotides, which are known to be determinant for the development of the gastrointestinal tract and immune function. The problem is caused by the conflict between the physiological timing of gastrointestinal tract development and the trend to intensify animal production by reducing the number of days spent by the newborn pig with the sow. Previous studies showed that supplementation of weaned pig diet with product containing the composition of nucleotides of sow's milk led to lesser reduction in the villous heigh/crypt depth ratio as well as to a significant reduction of diarrhea and mortality as compared to control pigs. The results are important but do not clarify the biological mechanism involved, for instance the rebuilding of interstitial mucosa.



Samples of piglets mid jejunum underwent routine histological processing in paraffin wax. Blocks were cut in slices (5μm), deparaffined and labeled with specific sets of antibodies (Ab):

- Mitosis - anti-Ki-67-FITC- conjugated Ab (BD Pharmingen, San Diego, Ca, USA);
- Apoptosis - anti-Cpp32 fragment of active caspase 3 (DAKO, Glostrup, Denmark), secondary swine anti-rabbit FITC-conjugated Ab (DAKO);
- Autophagy – anti – MAP I LC3 (Santa Cruz Biotechnology, Santa cruz, CA, USA), secondary Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA);
- DNA damage - anti-p53-FITC-conjugated Ab (DAKO, Glostrup, Denmark);
- Counterstaining of cell nuclei was made with 7AAD.

At least 14 mucosa images were collected by FV-500 confocal microscope for each data point. Object recognition (automatically elimination of pixels under 50) was performed using the Microimage analysis software on the basis fluorescent intensity.

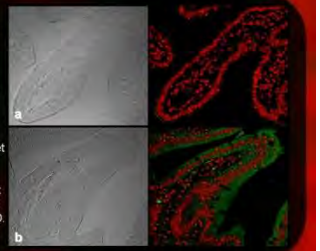


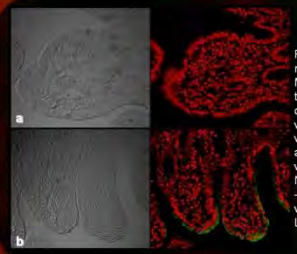
Mitosis

Fig. 1. Representative microphotographs of Ki67 expression (green fluorescence) in the crypts of mid jejunum in weaned piglets fed either with standard diet (a) or a diet supplemented (b) with nucleotides. Left – Nomarsky contrast, right – confocal image, nuclei were stained with 7-AAD. Lens magnification 40x.

Apoptosis

Fig. 2. Representative microphotographs of active caspase 3 expression in the upper two/thirds of the villi of mid jejunum in weaned piglets fed either with standard diet (a) or a diet supplemented (b) with nucleotides. Left – Nomarsky contrast, right – confocal image, nuclei were stained with 7-AAD. Lens magnification 40x.





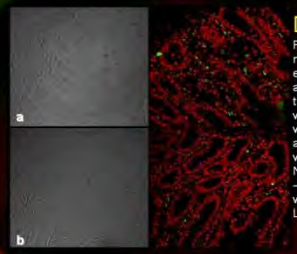
Autophagy

Fig. 3. Representative microphotographs of MAP I LC3 expression in the upper part of the villi of mid jejunum in weaned piglets fed either with standard diet (a) or a diet supplemented (b) with nucleotides. Left – Nomarsky contrast, right – confocal image, nuclei were stained with 7-AAD. Lens magnification 40x.

Table 1. The index of mitosis, apoptosis, autophagy and DNA damage, and mitosis/apoptosis ratio in the mucosa of the middle part of the jejunum in the control and dietary nucleotide supplemented piglets. Data are presented as mean and standard error of mean.

Indexes	Control	Nucleotide supplemented	P
Mitosis (%)	7.69±1.02	5.35±0.62	0.283 [b]
Apoptosis (%)	16.9±2.8	28.1±2.7	0.001 [a]
Mitosis/apoptosis ratio	0.454	0.191	
Autophagy (%)	4.51±1.22	5.14±0.99	0.042 [b]
DNA damage (%)	7.82±0.72	4.32±0.28	0.0001 [b]

[a] T-test, [b] Mann-Whitney test.



DNA damage

Fig. 4. Representative microphotographs of p53 expression in the crypts and lower one/third of the villi of mid jejunum in weaned piglets fed either with standard diet (a) or a diet supplemented (b) with nucleotides. Left – Nomarsky contrast, right – confocal image, nuclei were stained with 7-AAD. Lens magnification 40x.

CONCLUSIONS

- The mitosis/apoptosis index was lower in the supplemented group suggesting slower rebuilding of gut epithelium in these pigs as compared to the control.
- In the supplemented group a drop in p53 expression was observed, however, it remains uncertain whether the reduction in DNA damage index is due to the supplementation with nucleotides utilized as a source for repair processes, or due to the reduced DNA alterations.

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A novel cytometric approach to study intestinal mucosa rebuilding in weaned pigs fed with dietary nucleotides

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ABSTRACT

In the weaning period, transition from sow's milk to the post-weaning diet causes the withdrawal of important nutrients as milk nucleotides, which are known to be determinant for the development of the gastrointestinal tract and immune function. The role of dietary nucleotides in the rebuilding of gut epithelium remains unclear. The aim of the study was to evaluate using appropriate markers the rate of mitosis (Ki67) in the crypt epithelial cell, and the rate of apoptosis (active caspase 3), autophagy (MAP II LC3) and DNA damage (p53) in the crypt and villi epithelial cells of the small intestine of weaned pigs fed diets supplemented without/with nucleotides. An in-situ cytometry method, based on confocal imaging and automated quantitative analysis was implemented. The studies allowed us to understand molecular bases of animal performance which could not be assessed by a routine histometric approach. Namely, the dietary nucleotides provided more uniform small intestine epithelium with considerably less animal-to-animal variation in respect to mitosis (SEM = 1.02 in control vs. 0.62 in supplemented group), autophagy (SEM = 1.22 vs. 0.99) and expression of p53 protein (SEM = 0.72 vs. 0.28). A significant increase in ratio of apoptosis and autophagy and significantly lower p53 expression was found in the nucleotide-supplemented pigs as compared to control. The mitosis/apoptosis index was lower in the nucleotide-supplemented group suggesting slower rebuilding of gut epithelium in these pigs as compared to the control, respectively 0.454 to 0.191. Finally, in the supplemented group a drop in p53 expression was observed, however, it remains uncertain whether the reduction in DNA damage index is due to the supplementation with nucleotides utilized as a source for repair processes or due to the reduced DNA alterations.

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1. Introduction

Intestinal tissue poses a challenge for quantitative evaluation of growth and remodeling. Its complex architecture consists of a variety of cell and tissue types from different germ layers that tend to react in different way to the stimuli. In the present study we propose in-tissue cytometry, the quantitative automated method of evaluation of intestine mucosa remodeling based on the molecular markers of mitosis, programmed cell death (PCD) and DNA damage indexes. Microphotographs of immuno-labelled tissue sections were acquired by means of confocal microscopy and later quantitatively evaluated using automated image analysis software (Godlewski et al., 2007). At present, the most commonly accepted mitosis marker in the normal tissues is the expression of Ki67 protein which is required for cell progression through the division cycle as it organizes chromatin structure. It is expressed in

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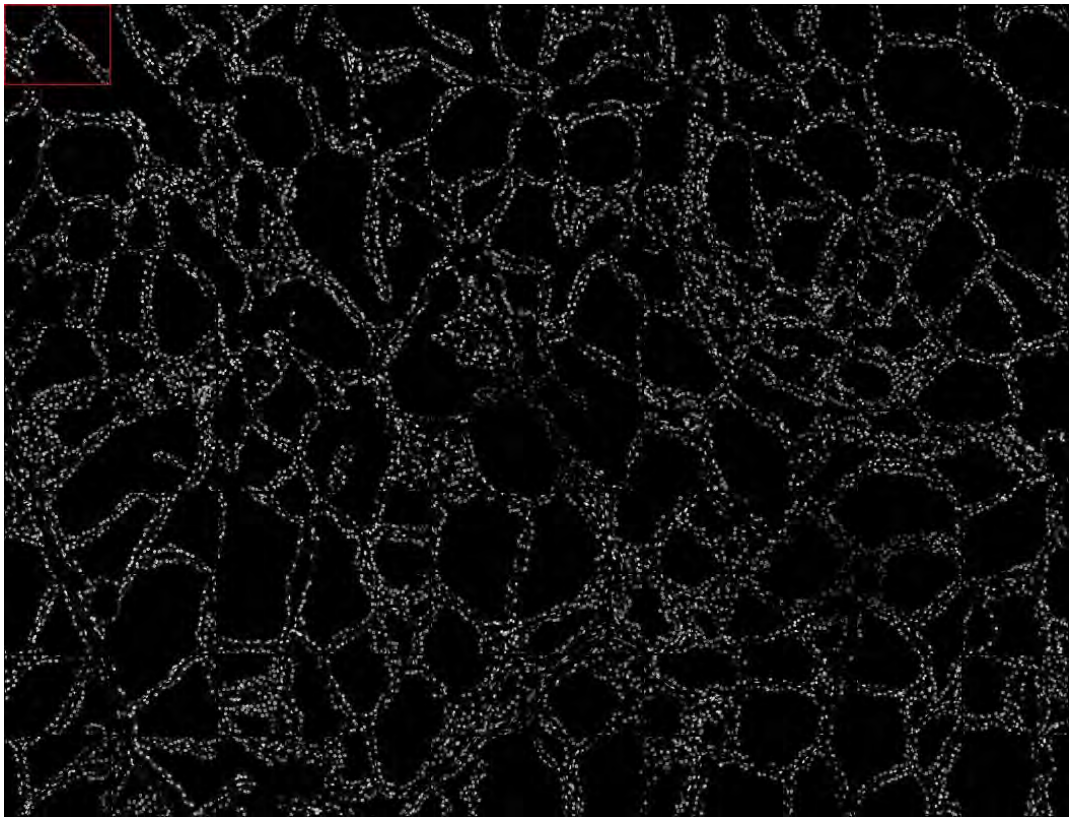
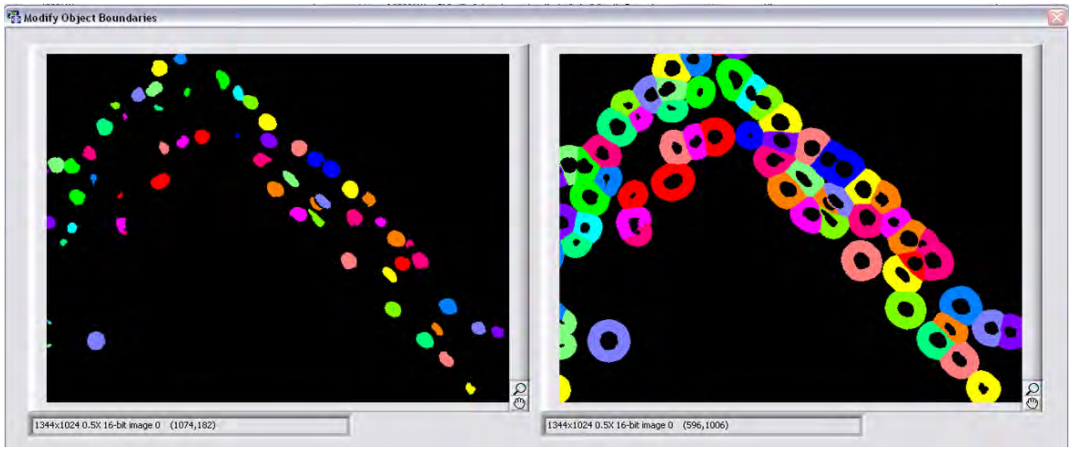
scanning cytometry

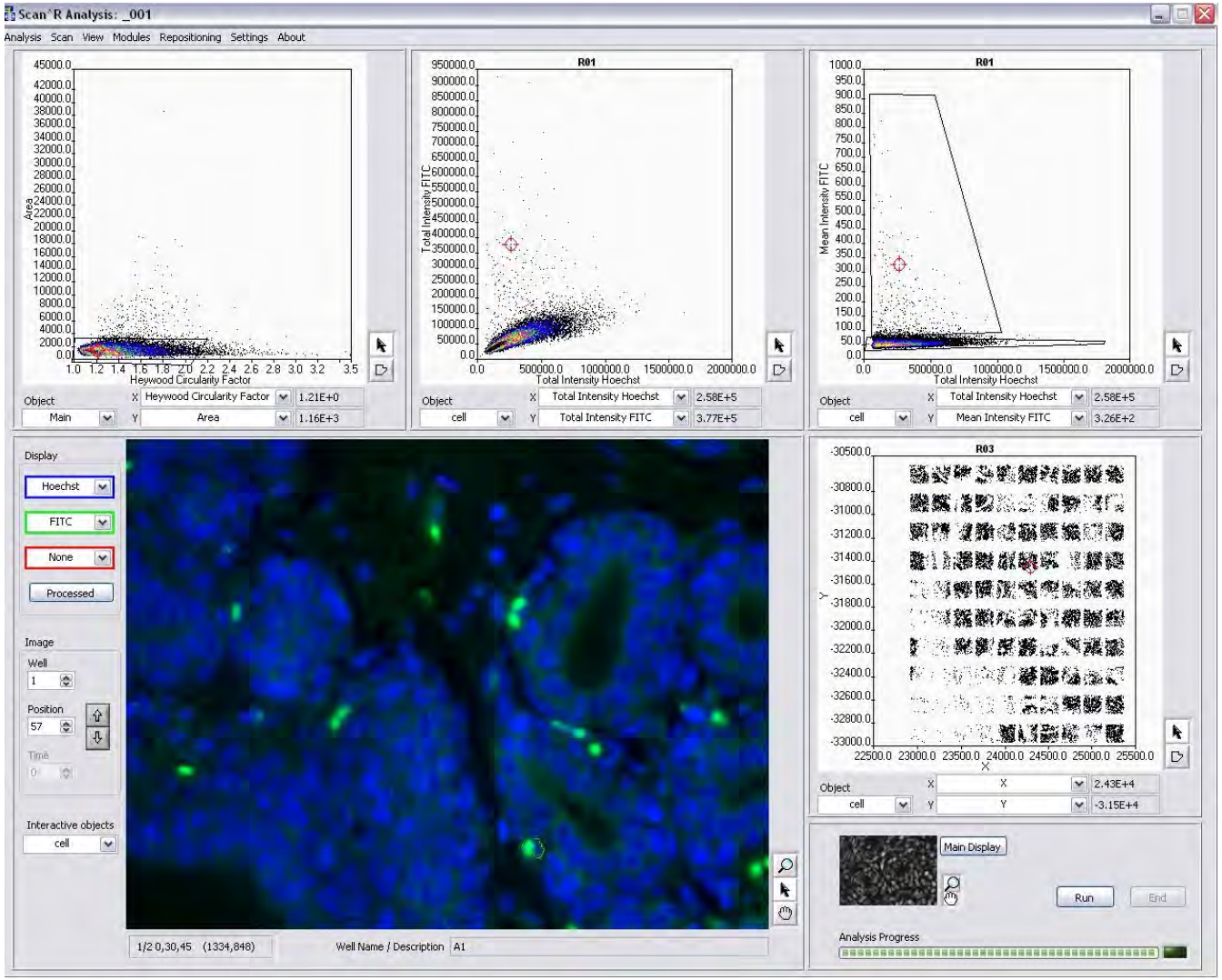
- Scan^R system





scanning cytometry

- Scan^R system



scanning cytometry

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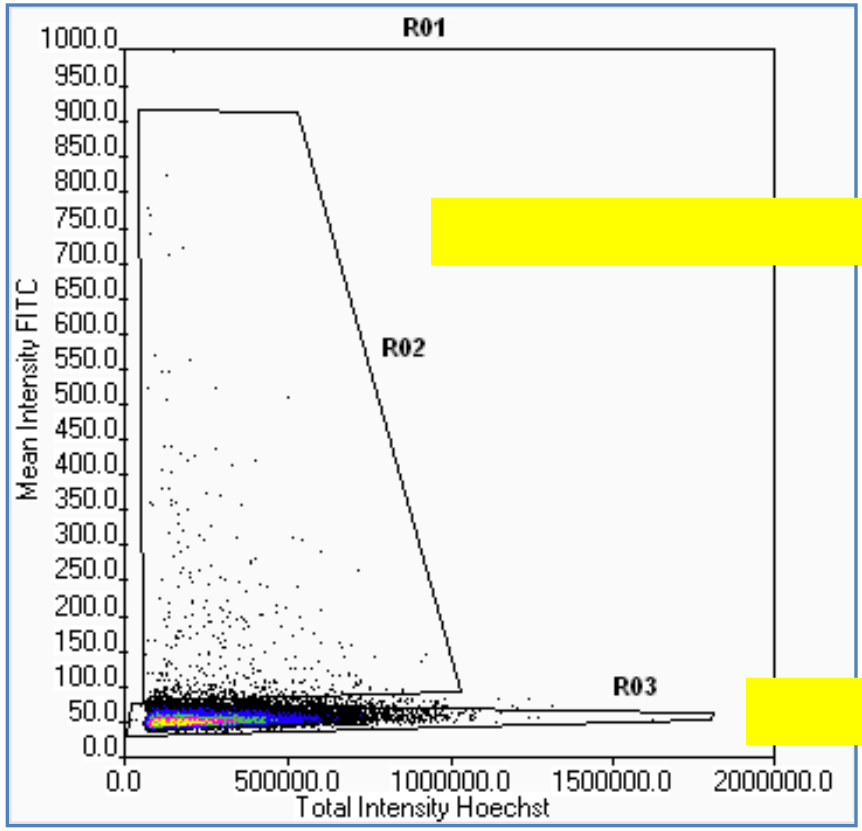
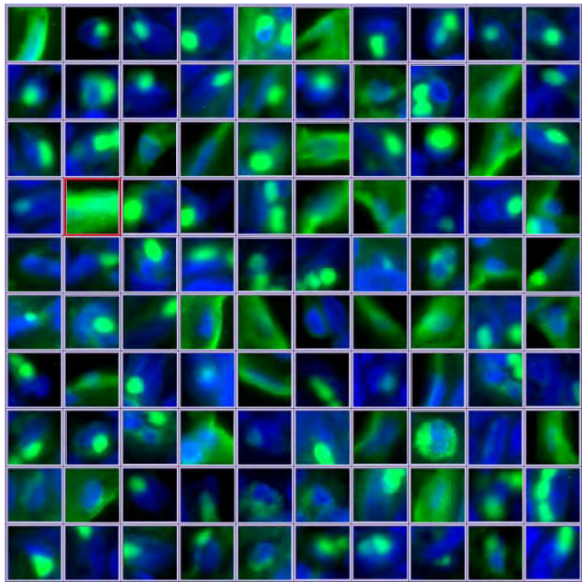
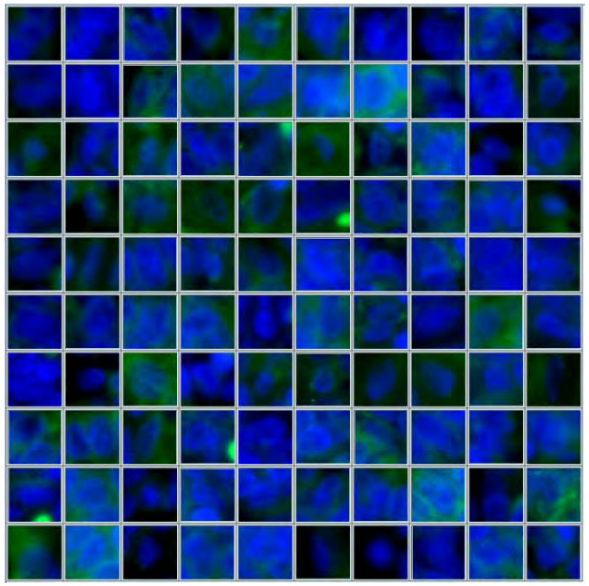


Sca-1 poz



Sca-1 neg



What about dairy cow stem cells? Semi – quantity and quantity evaluation of Sca1 expression in bovine mammary gland

Joanna Beata Bierła, Tomasz Motyl

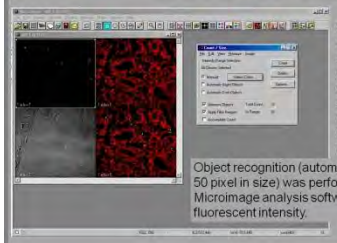
Department of Physiological Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences (SGGW), Poland

The comparison between stem cells presence in bovine mammary gland and visualisation of differentiation between stem cells and cells producing milk components is essential to acknowledge information concerning development of this specific epithelium Frequent repeating of life processes like proliferation, differentiation and involution during a life cycle make bovine mammary gland an unusual tissue. The basis for these changes seems to be stem cells, which were determined by us in our previous research by presence of expression of Sca1 in bovine mammary gland. Now we search for qualitative and quantitative estimation which could be the basis for analysis of their expression profile. Maybe we can find the answer why cells in bovine mammary gland are not transformed in carcinogenesis, because the frequency of tumors equals zero in this tissue. Therefore, it could be the basis for understanding fundamentals of carcinogenesis in woman's mammary gland.

How large is cells population with expression of Sca1 in bovine mammary gland ?

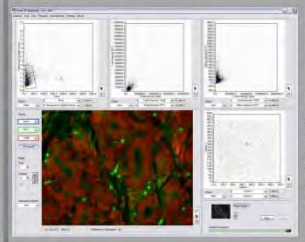
MATERIAL AND METHODS:

Mammary glands were obtained from 2 years old Holstein heifers. Samples were fixed in phosphate-buffered formalin, dehydrated, and paraffin embedded. The paraffin blocks were cut in slices (5 µm) which were mounted on silanized microscope slides (Sigma-Aldrich Chemie, Germany), deparaffined, permeabilized in 70% methanol (POCh, Poland) and next rinsed with PBS (Sigma-Aldrich Chemie, Germany).
Immunochistochemistry staining:
- mouse anti – Sca1 FITC – conjugated Ab (BD Pharmingen, USA), 1:200, 30 min in darkness,+4°C.
- cell nuclei were counterstained with 7-AAD (Sigma-Aldrich Corporation, USA)



Semi – quantity analysis
25 images (randomly acquired from the mammary tissue) were collected by FV-500 laser scanning confocal microscope (Olympus Poland) 60x NA 1.4 oil immersion lens.

Object recognition (automatically elimination under 50 pixel in size) was performed using the Microimage analysis software on the basis of fluorescent intensity.



Quantity evaluation
were done in Scan^R system (Olympus Poland)

There is possibility to count cells automatically using scanning cytometry Scan^R aqusition system vertically in the tissue slides. The obtained results are similar to flow cytometry and are analysed by Scan^R analysis system.

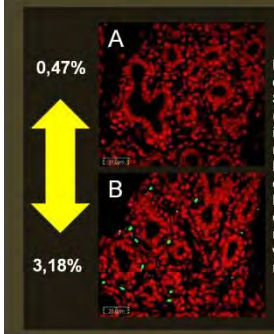


Fig. 1. Representative confocal images of Sca1 expression (green fluorescence) in the bovine mammary glands. A – heifres with low expression B –heifres with high expression nuclei were stained with 7-AAD. Lens magnification 40x.

RESULTS

Tab. 1. Results of quantity evaluation Sca-1 positive cells in heifer mammary tissue from Scan^R system (Olympus Poland). (n=5 sections per animal).

Parameter	Mean ± SD
Total number of analysed objects per section	16 987 ± 3 343
Number of objects with single nucleus (%)	95,24 ± 0,83
Objects Sca-1 positive (%)	2,14 ± 0,48

CONCLUSIONS

- inter-individual differences in presence of stem cells in bovine mammary gland based on Sca1 expression exists and can be used in animal selection
- both methods are able to quantitative analysis of Sca1 expression in mammary gland, but quantity evaluation is more valuable

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Identification, quantification and transcriptional profile of potential stem cells in bovine mammary gland

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ABSTRACT

Mammary gland is an organ with remarkable regenerative capacity apparent during successive reproductive cycles. It is implicated that this regenerative capacity is caused by existence of competent mammary stem cells (MaSCs), which provide for expansion of cell populations, cell renewal, and replacement. Although stem cell research has often focused on mammary development, information regarding bovine mammary stem/progenitor cell population is still scarce. The aim of our study was to estimate the size of bovine mammary gland stem/progenitor cell population using morphological, biochemical and molecular markers. Mammary glands of non-pregnant 2-year-old Holstein–Friesian heifers were used in the experiments. Quantitative analysis of stem/progenitor cells was done using flow and scanning cytometry. FACS analysis of Hoechst dye-effluxing side population (SP) revealed that the bovine mammary population of cells with stem-like characteristics amounts 0.46%. Additionally it was found that stem cell antigen-1 (Sca-1) is expressed on a small percentage of cells (2.14%) localized mainly in the basal layer of mammary epithelium. The Sca-1^{pos} cells do not express ER, but show down-regulation of genes connected with differentiation of mammary epithelium, and up-regulation of genes characteristic for cells of hematopoietic origin. Therefore we hypothesize that the Sca-1^{pos} cells found in bovine mammary gland can represent a population of cells of non-epithelial lineage, which inhabit the stem cell niche, enriching the stem/progenitor cell population in the mammary gland.

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1. Introduction

The mammary gland is a dynamic organ that undergoes significant developmental changes during postnatal life. It is composed of a branching network of ducts and lobuloalveolar structures comprised of mammary epithelium of two primary lineages: myoepithelial and luminal-epithelial. These two types of epithelial cells are presumed to arise from a common progenitor cell. The massive expansion of mammary epithelium is seen during puberty and pregnancy, preparing the gland for intensive milk production and secretion during lactation (Daniel and Smith, 1999). Remarkable regenerative capacity apparent during successive reproductive cycles implicates the existence of stem-like cells that enable the expansion, turnover and replacement of the cell populations in the mammary gland (Capuco and Ellis, 2005). The existence of mammary gland stem cells (MaSCs) was firstly demonstrated through transplantation experiments, which demonstrated that mammary epithelium could be serially transplanted into epithelium-free mammary fat pads generating functional mammary epithelial outgrowths (Daniel et al., 1968; Young et al., 1971; Smith and Medina, 1988; Wagner et al., 2002). Moreover, Kordon and Smith (1998)

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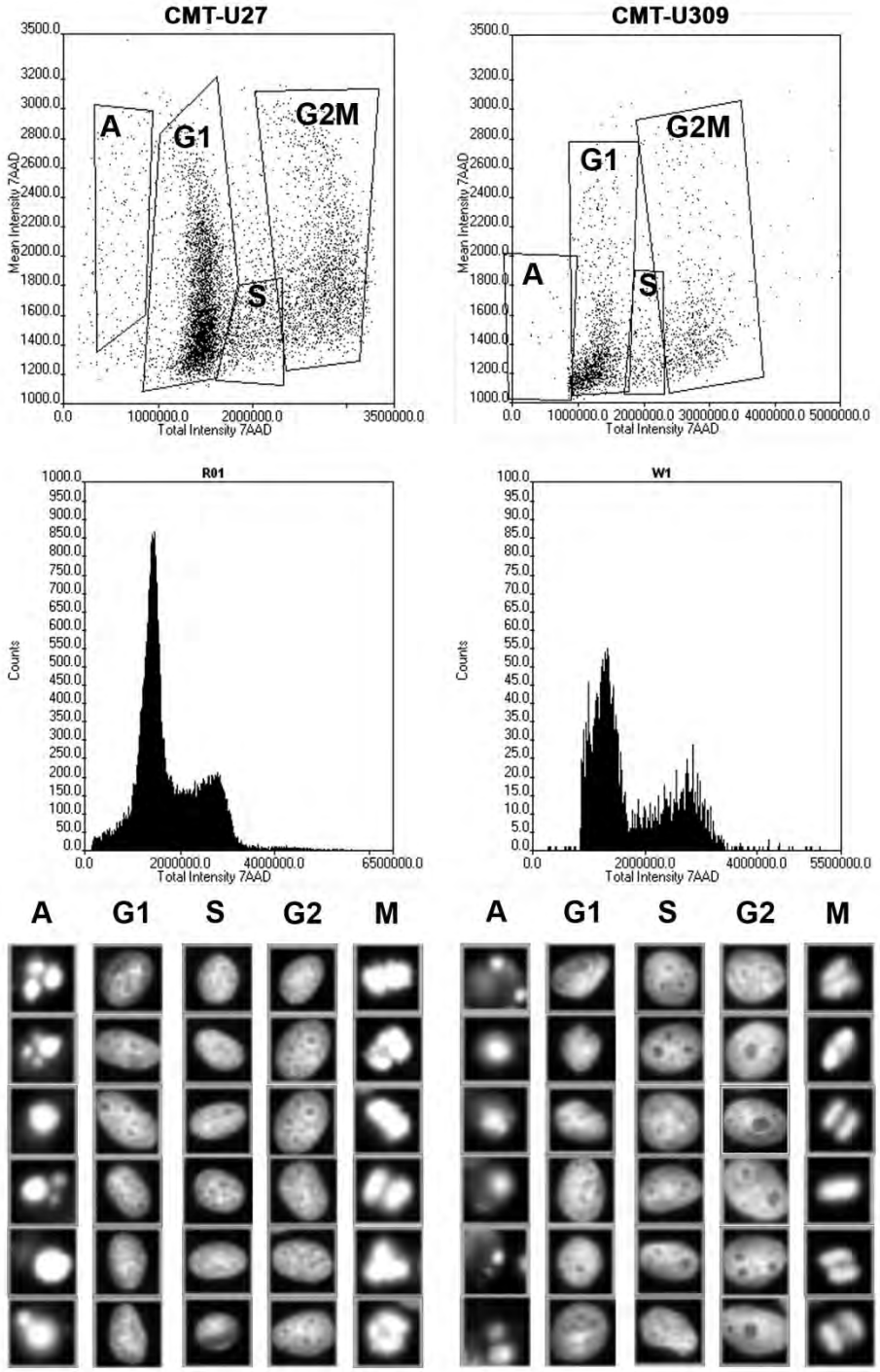
scanning cytometry

- Scan^R system



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Thank you for your attention!