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## Current methods of studying of the proliferative activity in experiment

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**Key words:** cell proliferation, immunohistochemistry, PCNA, antigen Ki67, Bromodeoxyuridine.

**Aims.** To analyze the contemporary scientific literature data about methods of determining of the proliferative activity, as well as the choice of an optimal method for its assessing in tissues with expected low proliferative activity.

**Methods and results.** This is necessary to select an optimal method for assessing of the proliferative activity in tissues with expected low proliferative activity. The article gives a detailed analysis of the current literature, describes various methods of studying of the proliferative activity of tissues in experiment.

**Conclusion.** Bromodeoxyuridine is a versatile and highly effective marker of DNA replication and cell proliferation, allowing carrying out a dynamic observation of the experiment, including tissues with expected low proliferative activity. It can be widely used in chronic experiments on small rodents, in the simulation of various pathologies and studying the effect of pharmaceuticals on the activity of proliferation.

### Сучасні методи дослідження проліферативної активності в експерименті

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З метою аналізу сучасних даних про методи визначення проліферативної активності, а також для вибору оптимального методу для роботи із тканинами з низькою очікуваною проліферативною активністю дослідили новітні публікації в галузі. Встановили, що підрахунок мітотичного індексу має низьку чутливість і специфічність, а такі методи, як імуноферментний аналіз, імуноблотинг, імуногістохімічне дослідження, полімеразна ланцюгова реакція мають високу чутливість та інформативність, але лише імуногістохімічні дослідження дають змогу візуалізації з можливістю топичної діагностики. Це свідчить, що, незважаючи на переваги і відмінності кожного маркера проліферації, застосування бромдезоксіуридину дає можливість здійснювати динамічне оцінювання та визначення проліферативної активності клітин у тканинах із низькою проліферативною активністю через його кумулятивний ефект.

**Ключові слова:** клітинна проліферація, імуногістохімія, PCNA, Ki67, бромдезоксіуридин.

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### Современные методы изучения пролиферативной активности в эксперименте

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С целью анализа современных данных о методах определения пролиферативной активности, а также для выбора оптимального метода для работы с тканями с низкой ожидаемой пролиферативной активностью исследовали новые публикации в отрасли. Установили, что подсчет митотического индекса имеет низкую чувствительность и специфичность, а такие методы, как иммуноферментный анализ, иммуноблоттинг, иммуногистохимическое исследование, полимеразная цепная реакция отличаются высокой чувствительностью и информативностью, но только иммуногистохимические исследования обеспечивают визуализацию с возможностью топической диагностики. Это свидетельствует, что, несмотря на преимущества и отличия каждого маркера пролиферации, применение бромдезоксидина позволяет проводить динамическую оценку и определение пролиферативной активности клеток в тканях с низкой пролиферативной активностью благодаря его кумулятивному эффекту.

**Ключевые слова:** клеточная пролиферация, иммуногистохимия, PCNA, Ki67, бромдезоксидин.

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Proliferation is an increasing of the body's tissues by cell multiplication by division. It may refer to the processes of different nature. Thus, cell proliferation underlies regenerative tissue neoplasms; proliferation observed in various hyperplasias; finally cell proliferation underlying tumor growth [Mitchison, 2003, Sulić et al., 2005]. Therefore, the identification and determining of the activity of proliferative processes is an important task of experimental and practical medicine.

**The aim** of this review was to analyze the contemporary scientific data about methods of determining of the proliferative activity, as well as the choice of an optimal method for its assessing in tissues with expected low proliferative activity.

There are many approaches to assess the proliferative activity. The proteins involved in cell cycle regulation have been opened within last decades. They may serve as

selectable markers of proliferating cells either in vivo, or in vitro. Determining of the proliferative activity is possible with using of different methods, such as counting of the mitotic index, enzyme immunoassay, immunoblotting, immunohistochemical analysis, PCR techniques. Lets consider some of them.

**Mitotic index.** Determining of the mitotic index is based on counting of the percentage of dividing cells of the total number of cells analyzed. The test tissue is treated with colchicine or its derivatives - substances that have properties to prevent the formation of microtubules, thereby preventing divergence of individual chromosomes in anaphase. After processing tissue the cell cycle stops in it, thereby enabling the identification of mitotic cells at the time of application of the drug [Прохорова и др., 2003]. The mitotic index (MI) calculated using the formula:

$$MI = (P+M+A+T)/N * 100,$$

where (P+M+A+T) is amount of cells at the stages of prophase, metaphase, anaphase and telophase, respectively, and N – the whole amount of cells analyzed.

The main disadvantages of this method are:

- low specificity;
- inability to assess the proliferative activity in the dynamics;
- inability to detect the proliferative activity in tissues with expected low proliferative activity.

Such methods as enzyme immunoassay, immunohistochemical analysis, immunoblotting, PCR techniques provide high sensitivity, specificity, informativity, but immunohistochemical techniques also provides visibility with the possibility of topical diagnosis. These techniques use monoclonal antibodies produced against specific antigens associated with cell proliferation. For today, there are methods of identifying of such proteins as cyclins, Ki-67, bromodeoxyuridine. We would like to elaborate on some of them.

*Proliferating cell nuclear antigen (PCNA)* is most commonly identified cyclin; it is an auxiliary protein of DNA polymerase delta. This molecule can be detected in human and animal paraffin-embedded or frozen tissue [Takahashi et al., 1993]. PCNA does not require pre-injection into the tissue under study, as confirmed by the positive results on archival samples, but it has some significant drawbacks:

- anti-PCNA immunohistochemistry may give a weak signal in non-proliferating tissues [Takahashi et al., 1993];
- the intensity of the signal depends on the method of fixing and amount of pre-heat treatment [Sasaki et al., 1992, Takahashi et al., 1993];
- it does not allow evaluating the proliferative activity characteristics and dynamics in tissues with expected low proliferative activity.

All steps in the application of this methodology held in vitro. The manufacturer recommends a 30-minute incubation period at room temperature. Formalin-fixed and paraffin-embedded tissue sections require unmasking of the antigen with high temperature treatment in 10 mM citrate buffer (pH 6.0) before immunostaining.

*Ki-67* antigen, also known as MKI67 - is another endogenous antigen [Scholzen, Gerdes, 2003], a nuclear protein that is bound and may be required for cell proliferation [Bullwinkel et al., 2003]. Furthermore, it is associated with transcription. Inactivation of the Ki-67 antigen leads to inhibition of the synthesis of ribosomal RNA [Bullwinkel et al., 2003, Rahmzadeh, 2007]. During the interphase Ki-67 can be detected only in the nucleus, whereas in mitosis most of the protein moves to the surface of the chromosomes. Ki-67 is present during all active phases of the cell cycle (G1, S, G2 and mitosis), but it is absent in resting cells (G0) [Scholzen, Gerdes, 2003]. This causes the possible variability of nuclear staining intensity. For detection in paraffin-embedded sections as well as in the case of PCNA thermal pre-treatment is required. Ki-67 can be detected in the tissue without its prior administration. However, it like as PCNA has several drawbacks:

- does not assess the features of the proliferative activity in the dynamics;

- does not assess the features of the proliferative activity in tissues with expected low proliferative activity.

Cell proliferation index Ki-67 was closely correlated with the index of cell proliferation of bromodeoxyuridine whether was not with PCNA, since the antigen Ki-67 has shorter half-life (1.5 - 2 hours), then with PCNA [Bromley et al., 1996], [Bologna-Molina et al., 2013]. All stages of the usage of this methodology held in vitro. The manufacturer recommends a 30-minute incubation period at room temperature or overnight at 4°C. The prior proteolytic processing of formalin-fixed and paraffin-embedded tissue sections is required. The antibodies react with the core antigen of humans and other mammals. Ki-67 protein initially was identified using prototype monoclonal antibodies, which were obtained by immunizing of mice with the nuclei of cells of Hodgkin's lymphoma [Gerdes et al., 1983]. However, currently MIB-1 monoclonal antibodies are exist; they are directed against another epitope of the same antigen. MIB-1 are used in clinical trials to determine the Ki-67 proliferative index. The main advantage of MIB-1 antibodies compared with the original Ki-67 antibodies (and the reason why they substantially replaced the original antibodies in clinical usage) is the possibility of using in fixed paraffin-embedded tissue samples after heat processing, unlike the original antibodies [Bánkfalvi, 2000].

*Bromodeoxyuridine (BrdU)* is an exogenous proliferation marker; it is synthetic nucleoside analogue of thymidine that is used for studying of the DNA replication [Lehner et al., 2011]. BrdU can be transmitted to daughter cells during the replication [Kee et al., 2002] and defined at least in 2 years after its application [Eriksson et al., 1998]. Enzymes (helicase, topoisomerase) and DNA-binding proteins unwind the DNA and hold the matrix in the untwisted state and rotate the DNA molecule. The correctness of the replication ensures with an exact match of complementary base pairs and DNA polymerase activity, able to detect and correct errors. Method's principle is based on the detection of BrdU that is capable to replace thymidine during replication, incorporating into new DNA [Lehner et al., 2011]. Anti-BrdU-antibody binds with BrdU, which has been exogenously administered into the newly synthesized DNA, enables the visualization of cells in which DNA replication occurs. The substance can get into the DNA only in S-phase of the cell cycle, thus eliminating the variability of staining [Lehner et al., 2011, Teruaki et al., 2011].

Features of this method are:

- it needs pre-injection into the tissue under study, but the exact timing and dosage of BrdU allows for evaluation of the proliferative activity in the dynamics;
- prolonged administration of BrdU can be used to assess the proliferative activity in tissues with expected low proliferative activity, since it will be detected in each molecule of the newly synthesized DNA after administration, and transmitted to daughter cells.

BrdU incorporated into the core is highly stable antigen, which gives a strong and reliable signal regardless of the method of fixation and further processing of tissues. Re-

Table 1

| Feature   | Methods       |                      |               |           |
|---|---------------|----------------------|---------------|-----------|
|   | Optical       | Immunohistochemistry |               |           |
|   | Mitotic index | PCNA                 | Ki-67         | BrdU      |
| Sensitivity   | High          | High                 | High          | High      |
| Specificity   | Low           | Medium               | High          | High      |
| Visibility  | Exist         | Exist                | Exist         | Exist     |
| Dynamic assessment  | Not exist     | Not exist            | Not exist     | Exist     |
| Assessment in tissues with expected low proliferative activity. | Not effective | Not effective        | Not effective | Effective |
| Staining variability  | High          | Medium               | Low           | Not exist |
| Specialized equipment   | No            | Required             | Required      | Required  |
| Prior administration  | No            | No                   | No            | Yes       |

search can be carried out on small rodents. Important qualities of this method are:

- low toxicity of the drug (approved for use in humans) [Takao et al., 1985, Takamitsu et al., 2006];
- minimally invasive administration, thus reducing the risk of death in experimental animals;
- the initial stage of the method is performed in vivo [Lehner et al., 2011].

BrdU does not affect the biochemical constants, neuroautonomic indicators and endocrine balance [Hoshino et al., 1985]. This is an important condition for maintaining of indicators of the simulated pathology. The second investigation phase conducted in vitro on conventionally made tissue sections, which can also be used to perform other histochemical studies. For successful immunohistochemical detection

of incorporated bromodeoxyuridine the DNA denaturation is required [Konishi et al., 2011].

For the integrated assessment of the methods above and decision of the most appropriate method for studying of the proliferative activity in the dynamics and in tissues with expected low proliferative activity we have compiled a table 1.

### Conclusions

Bromodeoxyuridine is a versatile and highly effective marker of DNA replication and cell proliferation, allowing carrying out a dynamic observation of the experiment, including tissues with expected low proliferative activity. It can be widely used in chronic experiments on small rodents, in the simulation of various pathologies and studying the effect of pharmaceuticals on the activity of proliferation.

### Список літератури

1. Прохорова И.М. Методические указания / И.М. Прохорова, М.И. Ковалева, А.Н. Фомичева; Ярослав. гос. ун-т. – Ярославль, 2003. – 16 с.
2. A comparison of proliferation markers (BrdUrd, Ki-67, PCNA) determined at each cell position in the crypts of normal human colonic mucosa / [M. Bromley, D. Rew, A. Becciolini et al.] // Eur. J. Histochem. – 1996. – Vol. 40. – № 2. – P. 89–100.
3. Bánkfalvi A. Comparative methodological analysis of erbB-2/HER-2 gene dosage, chromosomal copy number and protein overexpression in breast carcinoma tissues for diagnostic use / A. Bánkfalvi // Histopathology. – 2000. – Vol. 37. – № 5. – P. 411–419.
4. Cell Kinetic Studies of In Situ Human Brain Tumors With Bromodeoxyuridine / [H. Takao, T. Nagashima, J. Murovic et al.] // Rupp Cytometry. – 1985. – Vol. 6. – № 6. – P. 627–632.
5. Comparison of the value of PCNA and Ki-67 as markers of cell proliferation in ameloblastic tumors / [R. Bologna-Molina, A. Mosqueda-Taylor, N. Molina-Frecherо et al.] // Med. Oral Pathol. Oral Cir. Bucal. – 2013. – Vol. 18. – № 2. – P. 174–179.
6. Correlation Between Bromodeoxyuridine- Labeling Indices and Patient Prognosis in Cerebral Astrocytic Tumors of Adults / [F. Takamitsu, M. Matsutani, O. Nakamura et al.] // Cancer. – 2006. – Vol. 67. – № 6. – P. 1629–1634.
7. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation / [J. Gerdes, U. Schwab, H. Lemke, N. Stein] // Int. J. Cancer. – 1983. – Vol. 31. – № 1. – P. 13–20.
8. The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis / [N. Kee, S. Sivalingam, R. Boonstra, J. Wojtowicz] // Journal of Neuroscience Methods. – 2002. – Vol. 115. – № 1. – P. 97–105.
9. Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells / [J. Bullwinkel, B. Baron-Lühr, A. Lüdemann et al.] // J. Cell. Physiol. – 2006. – Vol. 206. – № 3. – P. 624–635.
10. Mitchison J. Growth during the cell cycle / J. Mitchison // International Review of Cytology. – 2003. – Vol. 226. – P. 165–258.
11. Neurogenesis in the adult human hippocampus / [P. Eriksson, E. Perfilieva, T. Björk-Eriksson et al.] // Nat. Med. – 1998. – Vol. 4. – № 11. – P. 1313–1317.
12. Proliferating cell nuclear antigen (PCNA) immunohistochemistry: Influence of tissue fixation, processing and effects of antigen retrieval / [H. Takahashi, Y. Oishi, T. Oyaizu et al.] // Micron. – 1993. – Vol. 24. – № 4. – P. 385–388.
13. Proliferating cell nuclear antigen (PCNA) immunostaining as an alternative to bromodeoxyuridine (BrdU) immunostaining for brain tumours in paraffin embedded sections / [A. Sasaki, H. Naganuma, R. Kimura et al.] // Acta Neurochir. (Wien). – 1992. – Vol. 117. – № 3–4. – P. 178–181.
14. Rahmanzadeh R. Chromophore-assisted light inactivation of pKi-67 leads to inhibition of ribosomal RNA synthesis / [R. Rahmanzadeh, G. Hüttmann, J. Gerdes, T. Scholzen] // Cell Prolif. – 2007. – Vol. 40. – № 3. – P. 422–430.
15. Scholzen T. The Ki-67 protein: from the known and the unknown / T. Scholzen, J. Gerdes // Cell. Physiol. – 2000. – Vol. 182. – № 3. – P. 311–322.
16. Deregulation of cell growth and malignant transformation / [S. Sulić, L. Panić, I. Dikić, S. Volarević] // Croatian Medical Journal. – 2005. – Vol. 46. – № 4. – P. 622–638.
17. The dark side of BrdU in neural stem cell biology: Detrimental effects on cell cycle, differentiation and survival / [B. Lehner, B. Sandner, J. Marschallinger et al.] // Cell and Tissue Research. – 2011. – Vol. 345. – № 3. – P. 313–328.
18. Visualization of Heavy Ion Tracks by Labeling 3'-OH Termini of Induced DNA Strand Breaks / [K. Teruaki, T. Akihiro, N. Toshiyuki et al.] // J. Rad. Research. – 2011. – Vol. 52. – № 4. – P. 433–440.

## References

1. Prohorova, I. M., Kovaleva, M. I. & Fomicheva, A. N. (2003) *Metodicheskie ukazaniya. Jaroslavl'* [in Ukrainian].
2. Bromley, M., Rew, D., Becciolini, A., et al. (1996) A comparison of proliferation markers (BrdUrd, Ki-67, PCNA) determined at each cell position in the crypts of normal human colonic mucosa. *Eur. J. Histochem.*, 40(2), 89–100.
3. Bankfalvi, A., Simon, R., Brandt, B., Burger, H., Vollmer, I., Dockhorn-Dworniczak, B., et al. (2000). Comparative methodological analysis of erbB-2/HER-2 gene dosage, chromosomal copy number and protein overexpression in breast carcinoma tissues for diagnostic use. *Histopathology*, 37(5), 411–419.
4. Hoshino, T., Nagashima, T., Murovic, J., Levin, E. M., Levin, V. A., & Rupp, S. M. (1985). Cell kinetic studies of in situ human brain tumors with bromodeoxyuridine. *Cytometry*, 6(6), 627–632.
5. Bologna-Molina, R., Mosqueda-Taylor, A., Molina-Frecherо, N., Mori-Estevez, A., & Sanchez-Acuna, G. (2013). Comparison of the value of PCNA and Ki-67 as markers of cell proliferation in ameloblastic tumors. *Medicina Oral Patologia Oral Y Cirugia Bucal*, 18(2), e174–e179.
6. Takamitsu, F., Matsutani, M., Nakamura, O., et al. (2006) Correlation Between Bromodeoxyuridine- Labeling Indices and Patient Prognosis in Cerebral Astrocytic Tumors of Adults. *Cancer*, 67(6), 1629–1634.
7. Gerdes, J., Schwab, U., Lemke, H., & Stein, H. (1983). Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *International Journal of Cancer*, 31(1), 13–20.
8. Kee, N., Sivalingam, S., Boonstra, R., & Wojtowicz, J. (2002). The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. *Journal of Neuroscience Methods*, 115(1), 97–105.
9. Bullwinkel, J., Baron-Lühr, B., Lüdemann, A., Wohlenberg, C., Gerdes, J., & Scholzen, T. (2006). Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. *Journal of Cellular Physiology*, 206(3), 624–635.
10. Mitchison J. (2003) Growth during the cell cycle. *International Review of Cytology*, 226, 165–258.
11. Eriksson, P. S., Perfilieva, E., Björk-Eriksson, T., Alborn, A., Nordborg, C., Peterson, D. A., et al. (1998). Neurogenesis In The Adult Human Hippocampus. *Nature Medicine*, 4(11), 1313–1317.
12. Takahashi, H., Oishi, Y., Oyaizu, T., Tsubura, A., & Morii, S. (1993). Proliferating cell nuclear antigen (PCNA) immunohistochemistry: Influence of tissue fixation, processing and effects of antigen retrieval. *Micron*, 24(4), 385–388.
13. Sasaki, A., Naganuma, H., Kimura, R., Isoe, S., Nakano, S., Nukui, H., et al. (1992). Proliferating cell nuclear antigen (PCNA) immunostaining as an alternative to bromodeoxyuridine (BrdU) immunostaining for brain tumours in paraffin embedded sections. *Acta Neurochirurgica*, 117(3-4), 178–181.
14. Rahmanzadeh, R., Hüttmann, G., Gerdes, J., & Scholzen, T. (2007). Chromophore-assisted light inactivation of pKi-67 leads to inhibition of ribosomal RNA synthesis. *Cell Proliferation*, 40(3), 422–430.
15. Scholzen, T., & Gerdes, J. (2000). The Ki-67 Protein: From The Known And The Unknown. *Journal of Cellular Physiology*, 182(3), 311–322.
16. Sulić, S., Panić, L., Dikić, I. & Volarević, S. (2005) Deregulation of cell growth and malignant transformation. *Croatian Medical Journal.*, 46(4), 622–638.
17. Lehner, B., Weidner, N., Aigner, L., Bauer, H., Brockhoff, G., Rivera, F. J., et al. (2011). The dark side of BrdU in neural stem cell biology: detrimental effects on cell cycle, differentiation and survival. *Cell and Tissue Research*, 345(3), 313–328.
18. Konishi, T., Takeyasu, A., Natsume, T., Furusawa, Y., & Hieda, K. (2011). Visualization of Heavy Ion Tracks by Labeling 3'-OH Termini of Induced DNA Strand Breaks. *Journal of radiation research*, 52(4), 433–440.

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