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Technical Note

# Successive Detection of Telomerase by IHC and Assessment of Telomere Length by Q-FISH in Paucicellular Cumulus Samples from Cumulus-Oocyte Complexes Obtained in Assisted Reproduction Programs

Anna A. Pendina [1], Mikhail I. Krapivin [1], Irina D. Mekina [1], Irina V. Aleksandrova [2], Yanina M. Sagurova [1], Evgeniia M. Komarova [1], Mariia A. Ishchuk [1], Andrei V. Tikhonov [1], Olesya N. Bespalova [1], Alexander M. Gzgzyan [1], Igor Yu. Kogan [1], Olga A. Efimova [1,*]

1. D.O. Ott Research Institute of Obstetrics, Gynecology and Reproductology, Mendeleevskaya Line, 3, St. Petersburg, Russia; E-Mails: pendina@mail.ru; krapivin-mihail@mail.ru; irendf@mail.ru; yanina.sagurova96@mail.ru; evgmkomarova@gmail.com; mashamazilina@gmail.com; tixonov5790@gmail.com; shiggerra@mail.ru; agzgzyan@gmail.com; ikogan@mail.ru; efimova_o82@mail.ru
2. St. Petersburg State University, Universitetskaya emb., 7/9, St. Petersburg, Russia; E-Mail: irina.ale98@mail.ru

* **Correspondence:** Olga A. Efimova; E-Mail: efimova_o82@mail.ru

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**Abstract**

This paper suggests an approach for the use of a single paucicellular histological sample to investigate two characteristics indicative of the cell's functional potential: 1) the content of telomerase reverse transcriptase (TERT) and 2) the telomere length. An algorithm has been suggested for the successive detection of the catalytic telomerase subunit with immunohistochemical staining and assessment of telomere length with Q-FISH on the same set of cells. The described approach uses three incontestable advantages of the FISH technique, namely, the possibility of analyzing target sequences in individual cells and paucicellular samples, the performance of several successive hybridization rounds with

different FISH probes on the same sample, and the examination of genomic regions consisting of repeats. This approach has been applied to assess the TERT content and telomere length in cumulus cells from human cumulus-oocyte complexes obtained from assisted reproduction programs. This approach provides an opportunity to examine the correlation between the TERT content and telomere length in cumulus cells surrounding the oocyte and the oocyte's capacity for fertilization, as well as the subsequent pre- and post-implantation development of the resulting embryo.

**Keywords**
Cumulus cells; methodological approach; IHC; TERT content; telomere length; Q-FISH

## 1. Introduction

The technique of fluorescent *in situ* hybridization (FISH) was developed and applied for the first time in the late 1960s [1]. FISH utilizes the ability of the DNA in the metaphase chromosomes and interphase nuclei from cytogenetic, cytological, and histological preparations to form highly resilient hybrid molecules with DNA(RNA) probes. The development and implementation of FISH have opened up unprecedented opportunities for the mapping of target sequences directly on the chromosomes and interphase nuclei of individual cells.

Over more than 50 years of its existence, the technique has undergone considerable improvement. The most significant change involves the transition from isotopic to non-isotopic detection using DNA(RNA) probes labeled with non-radioactive modified nucleotides [2]. This transition expanded the capabilities of the method, allowing the combination of DNA probes modified with various fluorochromes and the co-localization of two or more DNA sequences in an individual cell or on a single metaphase plate. It also contributed to a wider application of FISH in medical cytogenetics for the specification of structural chromosome rearrangements, identification of marker chromosomes, and investigation of chromosome mosaicism [3-7].

The early 21$^{st}$ century was marked by exponential growth in molecular genetic technologies, including Next Generation Sequencing [8-12]. However, despite the onslaught of new technologies and their high efficiency, FISH remains relevant for several tasks that are either impossible or challenging to perform using molecular genetic tools. Thus, FISH has several undeniable advantages. First, this technique detects the location and size of target sequences on individual chromosomes and cells, which provides the unique opportunity of working with minuscule samples or individual cells. Second, it facilitates the study of genomic regions that consist of repeats, which presents a major challenge for molecular genetic technologies. Third, the tool allows for a series of hybridization rounds with different probes on the same sample. In the new methodology presented in this study, we have utilized all these advantages.

We developed an approach to analyzing two essential parameters in the same set of cells: telomerase protein subunit (TERT) content and telomere length (TL). TL is a crucial characteristic of the genome. Extreme shortening of the telomere resulting from the mitotic activity and/or exogenous factors causes cell death [13]. Since telomerase can lengthen telomeres, its presence

may be important for the maintenance of the normal functioning of certain types of dividing cells [14, 15].

During ontogenesis, TL changes considerably due to both genetically programmed and exogenous factors [16-18], making the telomere among the more dynamic genome components and presenting a major obstacle to its investigation. An additional challenge for researchers is the DNA structure of telomeric regions, which consists of a varying number of tandemly repeated hexanucleotides [19].

In this study, we selected cumulus cells, which surround the oocyte and sustain its activity [20]. These cumulus cells enable the tropism of the oocyte through well-developed intercellular contacts, thus determining its functioning and potential for development [21]. The study of human cumulus cells is particularly relevant for the prediction of assisted reproduction outcomes, which are often hampered by challenges in the assessment of germ cell quality. Determining telomerase content and TL in cumulus cells could be instrumental to the development of novel approaches to assess oocyte quality and its potential for fertilization and preimplantation development. However, the main challenge of such an investigation is the small size of a cumulus cell sample, which is due to its separation from the cumulus-oocyte complex (COC). To correct this, we analyzed the telomerase content and TL with techniques such as immunohistochemical staining and quantitative fluorescence in situ hybridization (Q-FISH), which are the optimal tools for the analysis of paucicellular samples and individual cells. To increase the accuracy of Q-FISH and mitigate the impact of factors such as the efficiency of the hybridization process and the varying degrees of chromatin compaction, we normalized the measurement of fluorescent signal intensity. To that end, we obtained the ratios of the average telomere and the reference fluorescence intensity of probes. Relative TL analysis is sufficiently accurate, and in combination with the terminal restriction fragments (TRF) method, it is the gold standard for the assessment of TL, with a strong positive correlation of results [22].

Our approach includes the following stages: 1) the sampling of a cumulus fragment distal from the oocyte, 2) its transfer to a biopsy filter, 3) obtaining histological sections, 4) immunohistochemical (IHC) staining, 5) acquisition of digital images and registration of cell coordinates on the preparation, 6) Q-FISH analysis with telomeric probes, 7) digital imaging, and 8) FISH analysis with a reference probe (Figure 1). Each stage was performed sequentially on a single cumulus sample fixed on a glass slide. This approach facilitates the assessment of multiple parameters across the same set of cumulus cells, thus presenting a complete picture of their functional status and enabling a more accurate prediction of the oocyte's development potential.

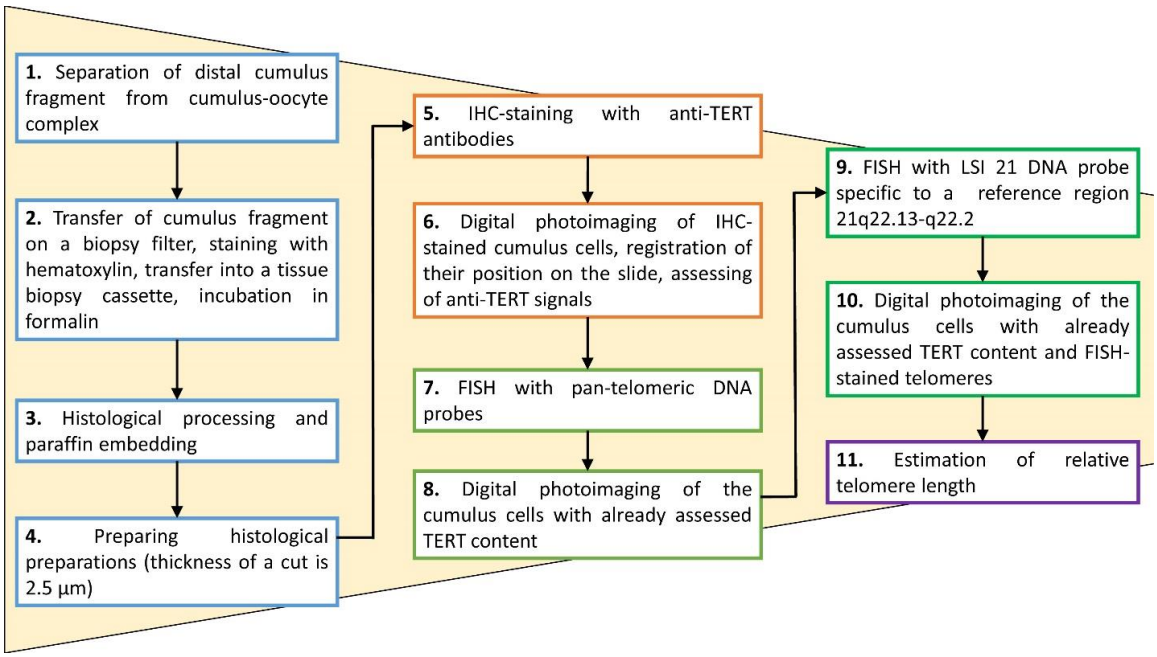


**Figure 1** Study design for the analysis of the protein subunit of telomerase reverse transcriptase (TERT) content and relative telomere length (TL) in cumulus cells.

## 2. Protocol and Guide Notes

### *2.1 Acquisition and Formalin Fixation of Cumulus Cell Samples from COCs*

In routine IVF/ICSI procedures, a portion of distal cumulus cells not adjacent to the oocyte is separated from the COC. The separated cumulus cells are not used in assisted reproduction and, therefore, can be enrolled in experimental research.

We used the following procedure to collect cumulus cell samples:

- Follicular fluid obtained by follicle puncture was transferred from the tube to a Ø 90 mm Petri dish;
- Follicular fluid was screened for COCs using a binocular microscope (magnification 6.5×);
- To release COCs from blood, they were transferred to a Ø 35 mm Petri dish with the gamete buffer G-MOPS (Vitrolife);
- The COCs were transferred to fresh G-MOPS buffer;
- Distal cumulus cells were mechanically separated from the COC in the G-MOPS buffer. For this, an insulin syringe needle was used to immobilize the sample, while another was used to cut the distal part of the cumulus. Thus, two fragments were obtained: an oocyte with adjacent cumulus cells and a distal cumulus fragment (Figure 2);
- The distal cumulus fragment was transferred to a biopsy filter (DiaPath) with a small pore diameter, which prevented the sample from diffusing in the solution during histological processing;
- The cumulus cells were stained using 2–3 drops of hematoxylin applied to the biopsy filter and covered with a second biopsy filter;
- Biopsy filters with cumulus cells were placed into tissue biopsy cassettes and incubated in buffered 10% formalin solution (pH = 7.2–7.4) at room temperature for 24 h.

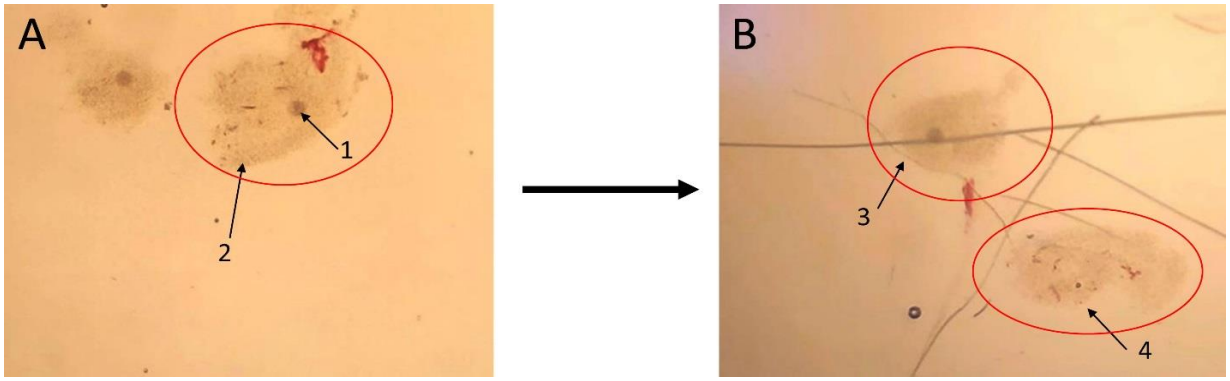


**Figure 2** Separation of a cumulus fragment from the cumulus-oocyte complex (COC), as obtained in the assisted reproduction program. A - COC (1 - oocyte, 2 - cumulus); B - two fragments obtained by the separation of COC: 3 – fragment containing the oocyte and adjacent cumulus cells, 4 – the distal cumulus fragment used for research. Magnification 6.5×.

### *2.2 Histological Processing of Cumulus Cell Samples*

Histological processing aims to conserve tissue morphology through dehydration, defatting, and subsequent paraffin embedding. Paraffin types differ in density and polymer content, which is crucial for histological processing. Type 1 and 3 paraffin have the least polymer content, which increases their ability to penetrate the tissue and diffuse into it. Type 9 paraffin has high polymer content and high density, which enables good quality microtomy. During histological processing, a certain tissue density is reached to further obtain quality preparations. If the density of the sample is insufficient, microtomy may be accompanied by tissue folding, ruptures, and other artifacts, which make accurate analysis impossible. Histological processing includes several steps of dehydration using ethanol and isopropanol supplemented with the surfactant Triton X15 to facilitate penetration of the tissue. After dehydration is complete, the sample is embedded in paraffin and placed into a tissue cassette.

During the first step of histological processing, tissue cassettes with cumulus cells were washed in flowing water for 30 min to remove traces of formalin. Then, the cassettes were placed in the Logos J histoprocessor with microwave function (Milestone, Italy). This technology facilitates rapid processing with minimal heating effects, thus, preserving the DNA and proteins in the sample.

The following protocol was used for the histological processing of cumulus cell samples:

- Incubation in 60% ethanol for 3 min at room temperature;
- Incubation in 95% ethanol at room temperature, with two changes after 2 min each;
- Incubation in 95% ethanol for 25 min at 65°C achieved by electric and microwave heating;
- Incubation in isoprep histological processing solution (absolute isopropanol 99.7% supplemented with Triton X15) for 72 min at 68°C achieved by electric and microwave heating;
- Incubation in Paraffin Type 3 (ThermoFisher Scientific) for 75 min at 82°C achieved by electric heating;
- Embedding cumulus cell samples in Paraffin Type 9 (ThermoFisher Scientific) and subsequent cooling using the CryoConsole (-4°C) up to paraffin solidification.

### *2.3 Preparations from Paraffin-embedded Cumulus Cells*

Tissue sections of 2.5 µm thickness were cut from paraffin blocks using a rotary microtome HistoCore MULTICUT (Leica Biosystems) and placed in a water bath at 37°C for a better spread. A thickness of 2.5 µm is optimal as it provides the maximum number of cumulus cells from a small sample. Then, the cuts were placed on positively-charged «Polysine» glass slides (Epredia) and incubated for at least 12 h at 37°C for optimal adhesion on glass and the vaporization of residual water.

### *2.4 IHC of Cumulus Cells with Antibodies Against the Telomerase Catalytic Subunit (TERT)*

IHC is a microscopic technique for the study of tissues, which involves the observation of certain antigens following the incubation of cuts with specific antibodies. This technique facilitates the detection of protein molecules in the studied tissue samples. Therefore, we used IHC for the detection of TERT in cumulus cells. To recover the reactivity of antigens in formalin-fixed paraffin-embedded cumulus cells, heat-induced retrieval of epitopes was performed using a citric buffer. Fluorescent staining for antibody detection allows the re-use of the same preparations for FISH. This is critical because only successive IHC and FISH provide information on both TERT content and TL in the same cell (Figure 3).

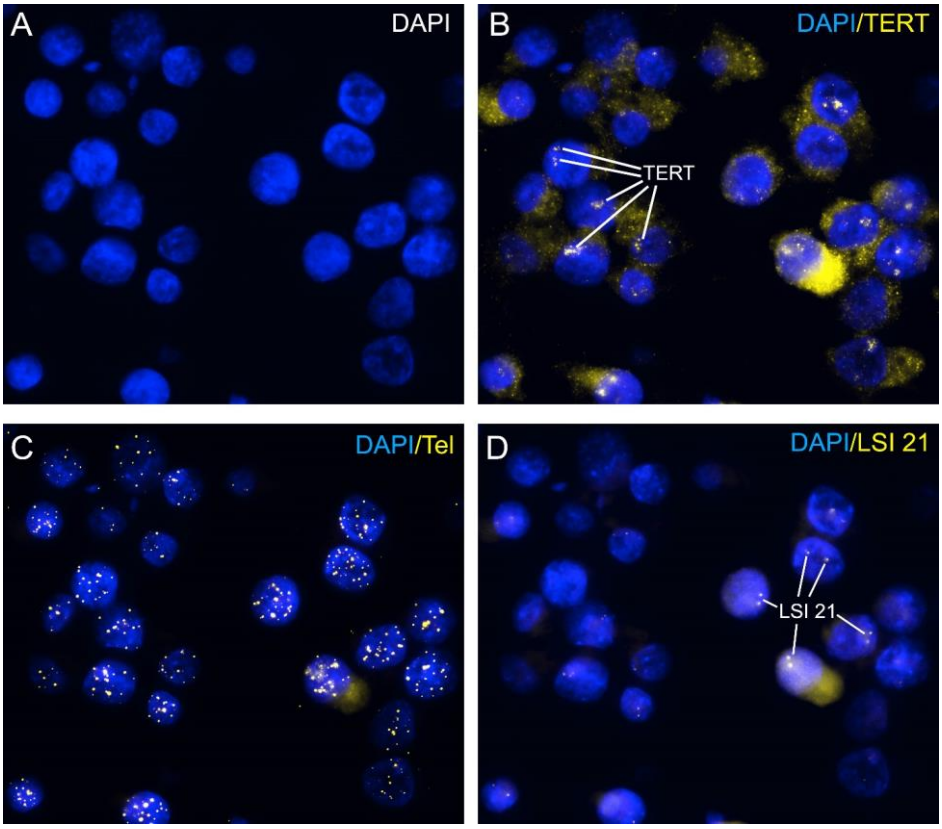


**Figure 3** (A) Cross section of cumulus cells after staining with DAPI; (B) immunohistochemical detection of telomerase reverse transcriptase (TERT); (C) FISH with pan-telomeric probes; (D) FISH with DNA probe for LSI 21 (21q22.13–22.2).

We used the following protocol for TERT IHC in cumulus cells:

- The slides with cumulus cell cuts were incubated for 30 min at 65°C for better adhesion of the cuts on the glass;
- Heat-induced retrieval of the epitope was performed using Lab Vision PT Module (Thermo Fisher Scientific) in a citric buffer (pH = 6.0) (Thermo Fisher Scientific) for 30 min at 98°C;
- The slide preparations were rinsed thrice using distilled water;
- The slide preparations were incubated in phosphate-buffered saline (1× PBS, pH = 7.2–7.4) for 30 min at room temperature;
- Then, 100 µL of blocking solution (1% bovine serum albumin in 1× PBS supplemented with 0.1% Tween 20) was applied to each slide preparation for 40 min at 37°C in a humidified chamber;
- Then, 100 µL of primary anti-TERT antibodies (Merck/Millipore, clone 7D5.2) diluted in the blocking solution (1:200) was applied to each slide preparation for 18 h at 4°C in a humidified chamber;
- The slide preparations were washed thrice using 1× PBS supplemented with Tween 20 (1:500) (Sigma Aldrich) for 3 min each at 37°C in a water bath with shaking;
- Then, 100 µL of secondary goat anti-mouse Alexa 555-conjugated antibodies (Thermo Fisher Scientific) diluted in blocking solution (1:500) was applied to each slide preparation for 2 h at 37°C in a humidified chamber;
- The slide preparations were washed thrice with 1× PBS supplemented with Tween 20 (1:500) (Sigma Aldrich) for 3 min each at 37°C in a shaking water bath;
- The slide preparations were rinsed with 1× PBS and distilled water;
- The slide preparations were air-dried at room temperature;
- The slide preparations were dehydrated in a series of ethanol concentrations (70, 80, and 96%) for 3 min each;
- The slide preparations were air-dried at room temperature;
- Finally, the slide preparations were mounted in DAPI-containing Vectashield antifade (Vector Laboratories, H-1200) and covered using coverslips.

### *2.5 Microscopic Analysis*

The cells were detected using a Leica DM 2500 fluorescent microscope. Digital photo imaging of cumulus cells with the registration of their position on the slide was performed using a Leica DFC345 FX camera and Leica Application Suite V.3.8.0 software. This procedure was necessary to find these cells after FISH.

### *2.6 FISH using PNA Probes for Telomeric Regions and with a Reference Chromosome Region during the Preparation of Cumulus Cells*

Telomeric regions were detected by quantitative fluorescence *in situ* hybridization (Q-FISH), which is a highly accurate technique and is optimal for single-cell analysis. It is the only approach that allows the assessment of TLs in the cells with already immunohistochemically determined TERT content. The intensity and size of the fluorescence signals are measured using software such as Image J, which has the appropriate tools for such an analysis. To mitigate the impact of chromatin condensation levels, hybridization efficiency, and possible variation in the brightness of the source of fluorescence on the measurement results, it is preferable to measure relative TLs. To that end,

absolute telomere fluorescence values were divided by the fluorescence level of the reference region; in our case, the 21q22 region, which is characterized by low variability. Such an approach increases the accuracy of Q-FISH.

In our study, FISH follows IHC for TERT and digital photo imaging. For FISH with telomeric regions, a set of telomeric PNA probes (Telomere PNA FISH/Cy3; DAKO, Denmark) and buffered solutions were used. The following protocol was used:

- Coverslips were gently removed under flowing water from the slide preparations that were immunohistochemically stained for TERT;
- The slide preparations were rinsed in distilled water;
- The slide preparations were air-dried at room temperature;
- The slide preparations were dehydrated in a series of ethanol concentrations (70, 80, and 96%) that were pre-cooled to 4°C, for 3 min each;
- The slide preparations were air-dried at room temperature;
- A commercially available mixture of telomeric PNA probes (Telomere PNA FISH/Cy3; DAKO/Agilent) was applied to the slide preparations and covered with round coverslips. The coverslip diameter and the volume of the mixture depended on the area with cumulus cells in the preparation. If a coverslip with a diameter of 5 mm was enough to cover the area of interest, 0.25 µL of PNA probe mixture was applied. Similarly, for coverslips with Ø of 6 mm, 0.32 µL of PNA probe mixture was used, for Ø of 7 mm, 0.45 µL was used, for Ø of 8 mm, 0.6 µL was used, and for Ø of 9 mm, 0.8 µL was used.
- The coverslips were sealed with rubber cement and placed in a Thermobrite hybridizer (Abbott laboratories);
- Denaturation was performed for 10 min at 88°C;
- Hybridization was performed for 18–20 h at 37°C;
- After hybridization was complete, the rubber cement was gently removed using thin forceps;
- To discard the coverslips, the slide preparations were incubated in the rinse solution (DAKO/Agilent) from a Telomere PNA FISH set, at room temperature for 1–2 min;
- The slide preparations were incubated in wash solution (DAKO/Agilent) from a Telomere PNA FISH set, in a shaking water bath at 62.5°C for 5 min;
- The slide preparations were rinsed in distilled water and air-dried at room temperature;
- The slide preparations were dehydrated in a series of ethanol concentrations (70, 80, and 96%) that were pre-cooled to 4°C, for 3 min each;
- The slide preparations were air-dried at room temperature;
- The slide preparations were mounted in a DAPI-containing Vectashield antifade (Vector Laboratories, H-1200) under coverslips and stored in a refrigerator until use for microscopy.

During further fluorescent microscopy, cumulus cells could be easily located based on the information on their position on the slide registered during the previous step. Digital photo imaging of the cumulus cells with previously assessed TERT content facilitates the analysis of both TLs and TERT in each cell. To increase the accuracy of the analysis, image acquisition options should be consistent across all photos obtained through one filter cube. While selecting the image acquisition options, saturated fluorescence signals should be avoided. Calibration for background fluorescence should also be performed through the software used for fluorescence analysis.

FISH is the next step following telomeric FISH and photoimaging. This uses a reference DNA locus-specific (LSI) probe for the 21q22 region (Abbott Laboratories) located on the long arm of chromosome 21. The following protocol was used:

- Under flowing water, the coverslips were gently removed from the slide preparations that were stained for TERT and telomeres;
- The slide preparations were rinsed using distilled water;
- The slide preparations were air-dried at room temperature;
- The slide preparations were dehydrated in a series of ethanol concentrations (70, 80, and 96%) for 3 min each at room temperature;
- The slide preparations were air-dried at room temperature;
- A commercial solution with the reference DNA probe LSI 21 was applied to the slide preparations covered with round coverslips;
- The coverslips were sealed with rubber cement and placed in a Thermobrite hybridizer (Abbott laboratories);
- Denaturation was performed for 10 min at 78°C;
- Hybridization was performed for 18–20 h at 37°C;
- After hybridization, rubber cement was gently removed using thin forceps;
- To discard the coverslips, the slide preparations were incubated in 4× saline sodium citrate (4× SSC) supplemented with 0.1% Tween 20 (Sigma-Aldrich) in a shaking bath at 3°C for 1–2 min;
- The slide preparations were incubated in two changes of 4× SSC for 5 min each at 37°C in a shaking bath;
- The slide preparations were rinsed with distilled water and air-dried at room temperature;
- The slide preparations were dehydrated in a series of ethanol concentrations (70, 80, and 96%) for 3 min each at room temperature;
- The slide preparations were air-dried at room temperature;
- The slide preparations were mounted in DAPI-containing Vectashield antifade (Vector Laboratories, H-1200), covered with coverslips, and refrigerated until use for microscopy.

During further fluorescent microscopy, cumulus cells could be easily located based on the information on their position on the slide registered during the previous step (after the telomeric FISH). Digital photo imaging of the cumulus cells with pre-recorded telomeric FISH signals facilitates the analysis of relative TLs, by dividing the absolute telomere fluorescence values by the fluorescence level of the reference 21q22 region. To increase the accuracy of the analysis, image acquisition options should be consistent across all photos obtained through one filter cube. While selecting the image acquisition options, saturated fluorescence signals should be avoided. Calibration for background fluorescence should also be performed through software used for fluorescence analysis.

## 3. Concluding Remarks

The development of novel non-invasive techniques for the prognosis of the capacity of germ cells for fertilization and further pre- and post-implantation embryogenesis is crucial for assisted reproductive technologies (ART). Currently, despite the rapid development of the industry, the effectiveness of ART is still below par. The decrease in ART effectiveness results from failures at any

step, from the difficulty of fertilization to embryonic arrest at either the pre- or post-implantation stage. The only reliable and informative criterion for embryo assessment is the genetic balance, which is checked by preimplantation genetic testing (PGT). The widespread use of PGT increases the effectiveness of ART, although it also raises new questions regarding the reasons for the developmental arrest of genetically balanced embryos. The latter indicates the need for the development of new safe methods to assess the developmental potential of germ cells and embryos. Therefore, a study on cumulus cells from COCs retrieved after controlled ovarian hyperstimulation is vital for the development of new tools for the prognosis of ART results. Importantly, after COC retrieval, cumulus cells are separated from the oocyte because they are not essential for *in vitro* fertilization. This makes the cumulus available for research without disturbing the ART procedures. However, the lack of available cumulus cells poses a serious challenge for studies. In this study, we suggested specific protocols for the collection and processing of cumulus to solve this problem. The optimized conditions for the histological preparation of cuts facilitate their successive IHC and FISH staining, which in turn allows the analysis of the TERT content and TL in the same cells. TL plays a crucial role in cell homeostasis. Telomerase is a key enzyme that maintains TL in dividing cells. Thus, the assessment of telomerase content and TL is a potentially promising criterion for the evaluation of cumulus cell potential. FISH has already been applied to cumulus cells in a study by Benkhalifa et al. (2012) [23], which used cytogenetic preparations instead of histological cuts. On the one hand, such an approach prevents methodological challenges during the preparation of histological cuts from paucicellular cumulus samples. On the other hand, IHC cannot be performed on cytogenetic preparations, as cells are incubated in Carnoy's fixative, which causes the extraction of most proteins. Studies attempting to simultaneously assess telomerase activity and TL in cumulus cells used molecular approaches such as quantitative PCR for the evaluation of TL and TRAP (telomeric repeat amplification protocol) to evaluate telomerase activity [24-26]. Such techniques require culturing cumulus cells to obtain a sufficient amount of DNA [24, 27, 28]. Multiple cell divisions during culturing may shorten the telomere. The question regarding the restoration of telomerase complex components, including TERT, during culturing remains open: if not restored, each daughter cell will inherit only half of the TERT amount from the maternal cell. One should also account for the difference in the effect of the cumulus microenvironment *in vivo* and *in vitro*. Several studies combined into one sampled cumulus cells from different COCs that were retrieved from one patient [25], making it impossible to analyze inter-cell differences, inter-COC differences, and the effect of cumulus cell parameters on the development of an adjacent oocyte.

In summary, the suggested algorithm not only allows the analysis of paucicellular cumulus samples but also facilitates the assessment of several parameters in the same cells, thus, providing a basis for the development of a novel approach to evaluate the developmental potential of an adjacent oocyte.

**Author Contributions**

Dr. Anna A. Pendina and Dr. Olga A. Efimova designed the study; Dr. Anna A. Pendina, Mr. Mikhail I. Krapivin, Dr. Irina D. Mekina, Ms. Irina V. Aleksandrova, Mrs. Yanina M. Sagurova, Dr. Evgeniia M. Komarova, Ms. Mariia A. Ishchuk, Dr. Andrei V. Tikhonov, Dr. Olesya N. Bespalova, Dr. Alexander M. Gzgzyan and Dr. Igor Yu. Kogan collected samples and performed experiments; Dr. Anna A. Pendina, Mr. Mikhail I. Krapivin and Dr. Olga A. Efimova analyzed results and performed literature seacrh; Dr.

Anna A. Pendina and Mr. Mikhail I. Krapivin drafted the manuscript; Dr. Anna A. Pendina and Dr. Olga A. Efimova critically revised the manuscript for important intellectual content.

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**Competing Interests**

The authors have declared that no competing interests exist.

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