August 22, 2016

Dr. Nam Nguyen, Ph. D.

*Journal of Visualized Experiments*

Science Editor

Dear Dr. Nguyen,

I am resubmitting my revised manuscript entitled "Establishment of Proliferative Tetraploid Cells from Nontransformed Human Fibroblasts.

Revisions made according to the editorial and peer review comments are the followings. All changes are indicated by the underlines in the new manuscript.

Revisions according to the editorial comments:

1. Formatting:

Page 1, Line 32 in the old manuscript (55028\_R1\_062316.docx); "... necessary to analyze ... " ---> Page 1, Line 32 in the revised manuscript; "... requisite for analyzing ..."

Page 1, Line 33 in the old manuscript (55028\_R1\_062316.docx); "... not easy. " ---> Page 1, Line 33-34 in the revised manuscript; "... rather challenging."

Page 2, Line 56 in the old manuscript (55028\_R1\_062316.docx); "... in vitro ..." ---> Page 2, Line 56 in the revised manuscript; "... *in vitro* ..."

Page 8, Line 342 in the old manuscript (55028\_R1\_062316.docx); "... in vitro ... " ---> Page 9, Line 354 in the revised manuscript; "... *in vitro* ..."

2. Editing for grammatical errors:

Page 2, Line 62 in the old manuscript (55028\_R1\_062316.docx); "... cannot be easy ..." ---> Page 2, Line 62 in the revised manuscript; "... not easy ..."

Page 2, Line 71 in the old manuscript (55028\_R1\_062316.docx); "... of drug removal." ---> Page 2, Line 71 in the revised manuscript; "... following drug removal. "

Page 3, Line 89 in the old manuscript (55028\_R1\_062316.docx); "Some cell strain ..." ---> Page 3, Line 94 in the revised manuscript; "Some cell strains ..."

Page 3, Line 92 in the old manuscript (55028\_R1\_062316.docx); "... the days before ..." ---> Page 3, Line 97 in the revised manuscript; "... on the day before ..."

Page 7, Line 289 in the old manuscript (55028\_R1\_062316.docx); "... on dish surface." ---> Page 7, Line 300 in the revised manuscript; "... to dish surface."

Page 7, Line 302 - Page 8, Line 317 in the old manuscript (55028\_R1\_062316.docx) ---> Page 8, Line 313 - 329 in the revised manuscript; The second paragraph of the discussion were rewrote using complete sentences.

3. Additional details in the protocol

1.1 (How are cells prepared?) It depends on the purpose of the experiment. Although somebody may want to induce tetraploid cells from the cells he(she) originally established (from the patient etc.), the present protocol was described on the assumption that they induce tetraploid cells from the established cell lines which can generally be obtained from public or private cell banks. Therefore, I changed the word "prepare" to "obtain".

2.1.3, 2.2.5 (Is the drug-containing medium replaced?) Yes. I revised the sentence as follows. "Incubate cells in drug-free medium in ..." ---> "Replace DC-containing medium with drug-free medium and ...".

2.2.3 (How are cells counted?) I do not think it is necessary to specify the method of cell counting, because It depends on the available facilities in laboratories and it is a basic technique in cell biology. I revised the sentence as follows. "Count cell number by an appropriate method ...". (We usually use an automatic cell counter called "Coulter counter", and I will use this counter in the video if necessary.)

3.1 (How long are cells incubated with trypsin? How is it neutralized?) I revised the description as follows. "Harvest cells (...) by incubating cells with a solution containing 0.05% trypsin and 0.02% EDTA for 10 min at 37°C, and neutralize trypsin by medium containing 10% FBS." In addition, trypsin and FBS were added in the materials table.

3.4 (Please provide a citation for FACS.) I added a reference for measurement of DNA content by Darzynkiewicz et al. (Reference No. 11)

4.1 (Is this a new DC treatment or an additional treatment for cells already treated (from section 2)?) This treatment with DC is for arresting cells in metaphase to prepare chromosome slides and has no relation to DC treatment for induction of tetraploidy at section 2. I added words "to arrest cells in metaphase" at the end of the sentence.

4.3.2, 4.3.3 (Please provide a citation) I added references for chromosome analysis (Reference No. 12) and multicolor FISH (Reference No. 13).

4. Results:

Figure 1 (Please provide a scale bar) I added scale bars in the figure and a description regarding scale bars in the figure legend .

Figure 2. (Please label the y-axes of the plots.) I added labels on the y-axes of the histogram plots.

Revisions according to the reviewers' comments:

Reviewer #1:

Regarding technical variations to be applied for the different cell lines ---> As far as cell lines I confirmed to date, there are not so much technical variations, such as number of cells seeded after shake off or time of DC post-treatment, to establish tetraploid cells. Only one big difference is that TIG-1 cells can become almost completely tetraploid by continuous treatment with DC, whereas other cell lines need separation of mitotic cells by shaking off during the course of DC treatment to be tetraploid cells free of diploid cells. However, this difference has already been described in the protocol. Another difference might be the time required to arrest cells in mitosis before the shake-off. Slower growing cells may have to be treated with DC for longer time than faster growing cells to be arrested in mitosis. I added a description regarding this point at page 3, line 117-118 (2.2.2) in the revised manuscript.

Regarding a method for PDL measurement ---> I added descriptions regarding PDL measurement at page 2, line 86 - page 3, line89 in the revised manuscript.

Reviewer #2:

Page 2, Line 82 to 84 in the revised manuscript (1.2); The following sentence was added. "or any other cell culture medium suited for the cell type to be studied"

Page 4, Line 153 to 154 in the revised manuscript (3.3.1.1); I revised the description regarding a method for DNA analysis of unfixed cells as follows. "Wash cells with 40 mM citrate buffer containing 250 mM sucrose. Subsequently treat cells with 250 μL of 0.1% (v/v) Nonidet P40 and 30 μg/mL trypsin in 200 μL of 40 mM citrate buffer containing 250 mM sucrose for 10 min at room temperature."

Page 4, Line 173 to Page 5, Line 178 in the revised manuscript (3.4); I revised the description regarding DNA analysis with a flow cytometer as follows; "Analyze DNA content of the cells using a flow cytometer with a 488 nm Laser and red channel emission filter (Long pass > 610 nm). Analyze a reference sample prepared from genuine diploid cells at the same time to assess ploidy of the target cells. Alternatively, add fluorescence standard beads to assess the fluorescence intensity ratio of diploid cells vs. the beads. Use the `pulse height vs. pulse width` option of the flow cytometer to discriminate between single tetraploid cells and clumps of diploid cells11."

Reference: I added a reference paper by Darzynkiewicz et al. (Reference No. 11)