(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 March 2020 (26.03.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/061500 A1

(51) International Patent Classification:
C12N 5/07 (2010.01) *C12N 5/0797* (2010.01)
C12N 5/0789 (2010.01)

(21) International Application Number:
PCT/US2019/052231

(22) International Filing Date:
20 September 2019 (20.09.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/734,314 21 September 2018 (21.09.2018) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

(54) Title: USE OF CHLOROPLASTS FOR OXYGEN PRODUCTION IN CELL CULTURES

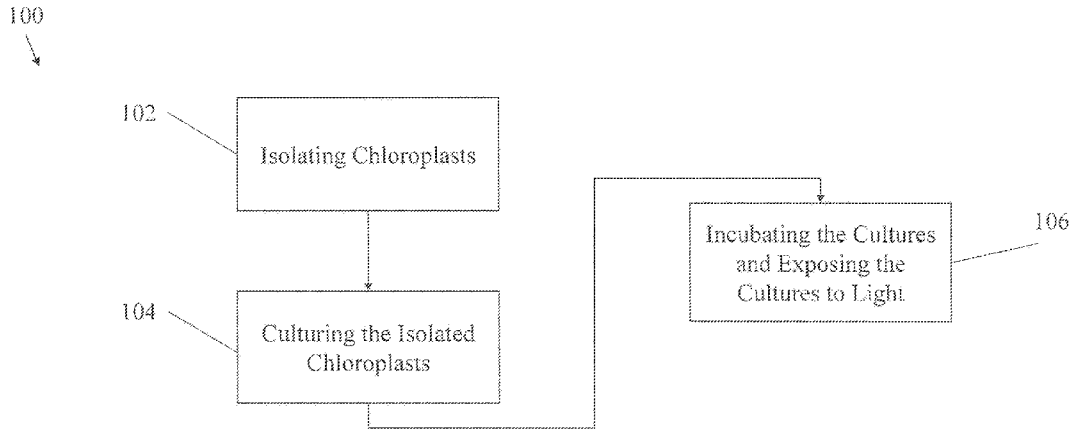

Fig. 1A

(57) Abstract: Methods and systems for increasing oxygen for use by mammalian cells are provided. In some embodiments, a method for increasing oxygen concentration for mammalian cells coculturing chloroplasts in at least one well plate with mammalian cells in a media and increasing oxygen production by exposing the chloroplasts to light.

WO 2020/061500 A1

[Continued on next page]

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

USE OF CHLOROPLASTS FOR OXYGEN PRODUCTION IN CELL CULTURES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and the benefit of United States Provisional Application No. 62/734,314, filed September 21, 2018, which application is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

[001] The disclosure relates generally to producing large quantities of oxygen for cell cultures and providing human cells with the ability to photosynthesize.

BACKGROUND

[002] Traditionally, there have been different methods used to isolate a wide variety of biological materials, including chloroplasts. For example, a mechanical support system can be used to allow for adherence of the isolated biological material, depending upon the type of material being isolated. After isolation, tradition methods can use the oxygen production from chloroplasts for energy production when introduced to metal. However, these systems and methods have failed to effectively incorporate isolated chloroplasts within human cell lines. There is a need to incorporate isolated chloroplasts within human cell lines to allow for improved oxygen production for a possible cell therapy or biotechnology production plant.

SUMMARY

[003] The present disclosure provides methods for increasing oxygen concentration of a cellular media or cells.

[004] In some aspects, the present disclosure provides a method for increasing oxygen concentration in a culture media, the method comprising culturing chloroplasts in a culture media including one or more mammalian cells, wherein the chloroplasts remain external to the mammalian cells in the culture media; and exposing the chloroplasts to light to cause oxygen production by the chloroplasts to enrich the culture media with oxygen to promote the growth or differentiation of the mammalian cells.

[005] In some aspects, the present disclosure provides a method for increasing oxygen concentration, the method comprising coculturing chloroplasts with one or more mammalian cells in a media, wherein the mammalian cells under conditions that cause the one or more

mammalian cells to take up the chloroplasts from the media; and increasing oxygen production in the mammalian cells by exposing the chloroplasts to light.

[006] In some embodiments, the methods can further comprise isolating chloroplasts from a source material. In some embodiments, the mammalian cells are human stem cells. In some embodiments, the chloroplasts are provided in a concentration of about 8 million chloroplasts per ml of media and about 12 million chloroplasts per milliliter of the culture media. In some embodiments, the chloroplasts are provided in a concentration of about 9 million chloroplasts per ml of media and about 11 million chloroplasts per milliliter of the culture media. In some embodiments, the chloroplasts produce between about 70 and 90% oxygen in the culture media.

[007] In some embodiments, the chloroplasts produce between about 80 and 90% oxygen in the culture media. In some embodiments, the method may further comprise maintaining viability of chloroplasts for at least about 72 hours. In some embodiments, the methods may further comprise maintaining viability of chloroplasts for between about 72 hours to about 120 hours. In some embodiments, the methods may further comprise isolating chloroplasts from a source material.

[008] In some aspects, the present disclosure provides a culture medium comprising chloroplasts in a concentration of about 8 million chloroplasts per ml of media and about 12 million chloroplasts per milliliter of the culture media. In some embodiments, the chloroplasts are provided in a concentration of about 9 million chloroplasts per ml of media and about 11 million chloroplasts per milliliter of the culture media. In some embodiments, the chloroplasts produce between about 70 and 90% oxygen in the culture media. In some embodiments, the chloroplasts produce between about 80 and 90% oxygen in the culture media. In some embodiments, viability of chloroplasts is maintained for at least about 72 hours. In some embodiments, viability of chloroplasts is maintained for between about 72 hours to about 120 hours. In some embodiments, the culture medium further comprises mammalian cells, wherein increasing oxygen production in the mammalian cells by exposing the chloroplasts to light.

BRIEF DESCRIPTION OF THE DRAWINGS

[009] The present disclosure is further described in the detailed description which follows, in reference to the noted plurality of drawings by way of non-limiting examples of exemplary embodiments, in which like reference numerals represent similar parts throughout the several views of the drawings, and wherein:

[0010] Figures 1A and 1B depict an example process for producing oxygen for cell cultures,

in accordance with the present disclosure;

[0011] Figures 2A and 2B depict images of isolated chloroplasts plated in wells, in accordance with the present disclosure;

[0012] Figure 3 depicts a graph showing increased oxygen concentrations in media including chloroplasts, in accordance with the present disclosure;

[0013] Figure 4A depicts an image of chloroplast cocultures after the addition of chloroplasts to the culture, in accordance with the present disclosure; and

[0014] Figures 4C and 4B depict images of chloroplast cocultures during the culture period, in accordance with the present disclosure.

[0015] While the above-identified drawings set forth presently disclosed embodiments, other embodiments are also contemplated, as noted in the discussion. This disclosure presents illustrative embodiments by way of representation and not limitation. Numerous other modifications and embodiments can be devised by those skilled in the art which fall within the scope and spirit of the principles of the presently disclosed embodiments.

DETAILED DESCRIPTION

[0016] The present disclosure discusses methods and systems for increasing oxygen concentration of cells cultures and cells using isolated chloroplasts. In some embodiments, the method involves extracellularly increasing oxygen concentration of human cell media by culturing isolated chloroplasts with the human cell media. In some embodiments, the method involves intracellularly increasing oxygen concentration of human cells via coculture of isolated chloroplasts with human cells, facilitating chloroplast uptake and sequestration within cells. By sterilely isolating chloroplast from plant materials and culturing them in human cell media, the chloroplasts can remain viable for over 48 hours or, in some embodiments, over 72 hours in culture and still produce significant amounts of oxygen for use by the human cells.

[0017] In some embodiments, the methods and systems of the present disclosure can be utilized to create temporary chloroplast-human cell symbionts that can conduct photosynthesis via coculture of isolated chloroplasts with human Mesenchymal Stem Cells (hMSCs). In some embodiments, such symbionts can uptake isolated chloroplasts and conduct photosynthesis *in vitro* and/or *in vivo*. Thereafter, the symbiotic chloroplast and human cells can be applied to different applications, such as for example, insertion into red blood cells for supporting mammalian cell growth or organism oxygenation. For example, when inserted into red blood

cells, chloroplast can produce and carry oxygen in addition to oxygen normally contained in the red blood cells (oxygen supplement). In addition to oxygen, chloroplasts can function to produce glucose, amino acids and fatty acids. In some embodiments, chloroplasts can be delivered by injection into human skin epithelial cells in vivo, providing nutrients for temporary nutritional supplementation.

[0018] In accordance with some embodiments of the present disclosure, a method for extracellularly increasing oxygen concentration of a cellular media is provided. The method includes sterilely isolating chloroplasts from a source material, culturing the chloroplasts in at least one well plate with a media, wherein the chloroplasts remain separate from other cells in the media, and incubating and exposing the cultured chloroplasts to light in the at least one well plate to increase oxygen concentration. The source material can be spinach leaves or other plant material.

[0019] In accordance with some embodiments of the present disclosure, a method for intracellularly increasing oxygen concentration of human cells media is provided. The method includes sterilely isolating chloroplasts from a source material, coculturing the chloroplasts in at least one well plate with human cell in a media, wherein the chloroplasts are taken up the human cells in the media, and incubating and exposing the cultured chloroplasts to light in the at least one well plate to increase oxygen concentration. The source material can be spinach leaves or another plant material. The human cells can be Mesenchymal Stem Cells (hMSCs) or dermal fibroblasts (hDF).

PROCESSING STEPS

[0020] Figures 1A and 1B depict example processes 100, 110 for implementing the methods of the present disclosure. Specifically, Figure 1A depicts a process 100 for isolating and culturing chloroplasts in well plates with media to extracellularly increase oxygen concentration of human cells media, as shown in FIGS. 2A and 2B. At step 102 chloroplasts are sterilely isolated from source materials. At step 104 the chloroplasts are plated in wells with a media. At step 106 the cultures (including chloroplasts) are incubated and exposed to light for a predetermined period of time. The results of process 100 is an increased dissolved oxygen concentration for the cells in the media. The resulting media can then be used for various medical implementations. In some embodiments, the chloroplasts alone can be used to oxygenate media.

[0021] Figure 1B depicts a process 110 for isolating and coculturing chloroplasts with human

cells to intracellularly increase oxygen concentration of human cells media. In some embodiments, such human cells can be Mesenchymal Stem Cells (hMSCs), human dermal fibroblasts (hDF), etc. In some embodiments, this can facilitate chloroplast uptake and sequestration within cells to increase oxygen concentration of human cells media. At step 112 chloroplasts are sterilely isolated from source martials and are aliquoted into well plates containing a medium. In some embodiments, the cells can be induced to take up chloroplasts in such a manner that the chloroplasts maintain its functions within a mammalian cell cytoplasm.

[0022] At step 114 the chloroplasts are co-cultured with cells (e.g., human cells) and are sequestered for a predetermined culture period well plates. The result of process 110 is the production of chloroplast-human symbionts for increased oxygen production. There are many benefits to this final product. For example, certain therapies require an oxygen source along with paracrine signaling from hMSCs, through creation of these symbiotes we should be able to achieve this in an acute non-toxic manner. Furthermore, this process could be applied to biotechnological production where oxygen levels are highly regulated. In some embodiments, chloroplasts can be co-cultured with mammalian cells, but not taken up by the mammalian cells.

SOURCE MATERIALS

[0023] The present methods can utilize different source materials for isolating chloroplast. In some embodiments, the chloroplast may be isolated from any combination of plants and algal. A variety of plants and algal, such as spinach, basil, arabidopsis, and tobacco, produce chloroplast and thus can be employed in the present disclosure. In some embodiments, the chloroplasts are isolated from spinach leaves.

CHLOROPLAST ISOLATION AND CULTURE

[0024] The isolated chloroplasts can be obtained from the plants or algal using any combination of methods. In some embodiments, the chloroplast can be isolated from the source material by deveining and finely chopping the source material with a fine blade (e.g., step 102 of process 100). The chopped source material can be placed into a mortar along with a grinding solution and ground with a pestle until a paste-like consistency. For example, the source material can be placed in 15 mL of grinding solution (0.33 M Sorbitol, 10 mM Sodium Pyrophosphate, 4 mM Magnesium Chloride, 2 mM Ascorbic Acid, pH was adjusted to 6.5). Thereafter, the paste can be filtered. For example, the paste can be filtered through two layers

of sterile cheesecloth and then spun down in a centrifuge at 300g for 1 minute at 4°C. Additionally, supernatant resulting from this process can be collected for further processing. For example, the supernatant can be collected and subsequently re-spun down in a centrifuge at 1000g for 7 minutes at 4°C. The supernatant after the second centrifuge cycle can be removed and a pellet containing the chloroplasts can resuspended in a suspension solution. In some embodiments, a pellet having chloroplasts can be created by centrifuging the solution with the chloroplasts. The pellet containing the chloroplasts can be, for example, suspended in 5 mL of suspension solution (0.33 M Sorbitol, 2 mM Ethylenediaminetetraacetic acid, 1 mM Magnesium Chloride, 50 mM HEPES, with the pH adjusted to 7.6). After processing, the isolated chloroplasts can be counted using a hemocytometer before being used in subsequent steps.

[0025] In some embodiments, sterile chloroplasts can be isolated from spinach leaves and are plated in a well, with a media therein, for a predetermined period of time for culturing (e.g., step 104 of process 100). For example, spinach cells can be plated in a well with a Mesenchymal Stem Cell Growth Medium (MSCGM) for three days, as discussed with respect to Figure 1A. Any cell culture medium can be used. In some embodiments, the medium may be buffered with HEPES, but not sodium bicarbonate, and will not include penicillin-streptomycin. Additionally, Gentamycin, but can be used as an antibiotic and Amphotericin B can be used as an anti-fungal. In some embodiments, normal mammalian cell culture medium including serums such as Fetal Bovine Serum can be used for these processes. In some embodiments, the chloroplasts can be cultured in MSCGM alone or with additional chloroplasts. Thereafter, the chloroplasts can be incubated and exposed to light (e.g., step 106 of process 100).

[0026] Once isolated, the chloroplasts can be used to prepare a culture medium. For example, the present disclosure provides a culture medium comprising chloroplasts in a concentration of about 8 million chloroplasts per ml of media and about 12 million chloroplasts per milliliter of the culture media.

[0027] Figure 2A depicts an image 200 taken at 20X enhancement showing freshly isolated chloroplasts 202, from a spinach leaf, plated in wells of a medium 204 at a concentration of 10 million chloroplasts 202 per mL of MSCGM medium 204. Similarly, Figure 2B depicts an image 200 taken at 20X enhancement showing freshly isolated chloroplasts 202, from a spinach leaf, plated in wells of a medium 204 at a concentration of 1 million chloroplasts 202 per mL of MSCGM medium 204. Within the images 200, 210, the isolated chloroplasts 202 remained

vibrant green and photosynthetically viable after isolation. In some embodiments, the chloroplasts remain viable for at least 72 hours. In some embodiments, the chloroplasts remain viable between 48 hours and 120 hours. In some embodiments, the chloroplasts can remain viable for between about 72 hours to about 120 hours. In some embodiments, the isolated chloroplasts 202 can be cultured in the plated well of medium 204 for a predetermined period of time to produce oxygen.

[0028] Figure 3 depicts a chart 300 showing how chloroplasts isolated from spinach leaf are able to increase oxygen concentration in media. In some embodiments, Mesenchymal stem cell media or DMEM with FBS can be used. In particular, Figure 3 depicts a bar graph showing a first bar shows the percentage of oxygen produced with media 204 alone (about 18%) and the second bar shows the percent of oxygen produced with the isolated chloroplast 202 (about 10 million chloroplasts) in the media 204 (about 82%). The 82% oxygen level can be produced from isolated chloroplast within two days after isolation.

[0029] In some embodiments, the media may be supplemented between about 6 million chloroplasts per ml of media and about 12 million chloroplasts per ml of media. In some embodiments, the media may be supplemented between about 8 million chloroplasts per ml of media and about 12 million chloroplasts per ml of media. In some embodiments, the media may be supplemented between about 9 million chloroplasts per ml of media and about 11 million chloroplasts per ml of media. In some embodiments, the chloroplasts can produce between about 70 and 90% oxygen. In some embodiments, the chloroplasts can produce between about 75% and 85% oxygen. In some embodiments, the chloroplasts can produce between about 80% and 90% oxygen. The chart in Figure 3 shows that the presence of chloroplasts in culture media leads to strongly significant increases in media dissolved oxygen levels over the isolated chloroplasts cultured alone in human cell media. This increase in oxygen increases the possible applications for the media, as discussed in greater detail herein.

CHLOROPLAST SYMBIONTS

[0030] In some embodiments, spinach leaves can be utilized to create temporary chloroplast-human cell symbionts 206 that can conduct photosynthesis via coculture of isolated chloroplasts 202 with human Mesenchymal Stem Cells (hMSCs) 208, or any cell type that actively digests small particles so that the cells can uptake isolated chloroplasts 202, which can then conduct photosynthesis *in vitro*.

[0031] To create chloroplast-human symbionts 206, a solution containing sterilely isolated chloroplasts 202 can be aliquoted into well plates containing hMSCs. There are no specific

steps or processes used for plating the chloroplasts in the wells, the hMSCs 208 and hDFs will inherently uptake the chloroplasts 202 when cocultured together. For example, within 30 minutes to 24 hours from the addition of chloroplasts to hMSC culture wells, chloroplasts will appear to be "naturally" and endosymbiotically incorporated into the cytoplasms of the hMSCs, remaining intracellularly sequestered until at least day 8 of the culture period. The relationship between chloroplasts and cells can be 4-7 chloroplasts per cell. After culturing, in some embodiments, the symbiotes can be exposed to light through modification of the incubators where light strips (e.g., light emitting diodes (LEDs)) were attached to the rack directly above the cultures and were turned on for 12-hour periods of time. Chloroplast-human cell symbiotes survive up to a week after cocultures begin.

[0032] Figures 4A-4C depict images 400, 410, 420 demonstrating that viable, photosynthetically-functional chloroplasts 202 are incorporated and sequestered within hMSCs (in some capacity) using the process 110 discussed with respect to Figure 1B. Figure 4A depicts an image 400, taken at 20X enhancement, of chloroplast-hMSC cocultures 206 24 hours after the addition of chloroplasts 202 to the culture 204. As shown in Figure 4A, chloroplasts 202 appear to be incorporated by hMSCs and residing within their cytoplasms. Similarly, Figure 4B depicts an image 410, taken at 20X enhancement, of chloroplast-hMSC cocultures 206 on day 4 of the culture period. As shown in Figure 4B, chloroplasts 202 have remained sequestered within hMSCs remain bright green and viable. Some hMSCs have morphologies resembling those of hMSC-derived chondrocytes. Figure 4C depicts an image 420, taken at 20X enhancement, of chloroplast-hMSC cocultures 206 on day 8 of the culture period. As shown in Figure 4C, hMSCs still appear to have sequestered chloroplasts 202 within their cytoplasms, and hMSCs continue to adopt a spherical morphology characteristic of chondrocytes. Thus, chloroplasts are able to be incorporated by hMSCs *in vitro* and remain sequestered and photosynthetically functional for at least 8 days after their uptake.

APPLICATIONS

[0033] There are a myriad of applications of photosynthetic human cells and efficient, cheap, and on-demand oxygen production by isolated chloroplasts is only expanding and currently unrecognized by the scientific community. Specifically, a lack of oxygen is a common problem that results in irreversible tissue death, and by having an isolated chloroplast population that could be delivered to such areas would open up a way to acutely treat such disorders. Furthermore, the combination of chloroplasts and hMSCs could act as a combinatory therapy where chloroplasts could provide oxygen and the hMSCs could provide paracrine signalling

which has been shown to have a positive regenerative affect. Chloroplasts also are non-toxic to mammalian cells and thusly could be incorporated into biomanufacturing of pharmaceuticals where oxygen level regulations are necessary to provide for positive manufacturing of the drug products. Additionally, the lack of money, time, and training with which oxygen concentration can be increased and photosynthesis can be induced using the chloroplast protocols discussed herein.

[0034] Specifically, there are several opportunities for both photosynthesizing chloroplast-human cell symbionts and isolated chloroplasts in culture. Specifically, isolated chloroplasts could be used to enhance cell cultures with oxygen production, to grow full organs or tissues *in vitro*, to vascularize and regenerate ischemic tissues, to quicken wound healing, to enhance human performance and physical ability, to produce oxygen in hypoxic environments (e.g. polluted environments or outer space), and to grow clean meat in vitro for consumption. Chloroplast-human cell symbionts and the process of endosymbiotic chloroplast uptake could be used to facilitate the destruction of cancerous tumours, to deliver biomolecules (e.g. insulin) or drugs via genetic engineering of chloroplast DNA and injection GM chloroplasts into human tissue, to quickly and easily bring about the differentiation of human stem cells to a certain fate, to function as an alternative to dangerous gene therapies, and to create permanent chloroplast-human chimeras that are healthier than normal humans due to adoption of a glycolytic metabolism. In some embodiments, the chloroplasts can be used for internal or external wound healing. In some embodiments, the chloroplasts may be delivered as part of a cream, ointment, or lotion that can be applied to an external wound. In some embodiments, a wound dressing impregnated with chloroplasts is provided. In some embodiments, the chloroplasts may be delivered to an internal wound using, for example, a biocompatible polymer.

[0035] The devices and methods of the present disclosure are described in the following Examples, which are set forth to aid in the understanding of the disclosure, and should not be construed to limit in any way the scope of the disclosure as defined in the claims which follow thereafter. The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the embodiments of the present disclosure, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be

accounted for.

EXAMPLES

[0036] Chloroplast Culture for Increased Media Oxygenation - Dissolved Percent Oxygen Data for Cultures

[0037] Chloroplasts were sterilely isolated from spinach leaves and plated in wells with zero, 1 million, 10 million, or 68.5 million chloroplasts per mL of Mesenchymal Stem Cell Growth Media (MSCGM) (Lonza, Walkersville, MD). In order to determine if chloroplasts could produce measurable amounts of oxygen after 3 days of dark incubation and 16 hours of light exposure, cultures' dissolved percent oxygen concentrations were measured with an SDR Oxygen Sensor (PreSens). Experimental cultures with 68.5 million chloroplasts in suspension solution (without antibiotics) had a dissolved oxygen concentration of 39.63% ± 3.02% in comparison to the 18.60 % ± 0.12% dissolved oxygen of the media-alone control group ($p = 0.001$). Even more significant, experimental cultures with 10 million chloroplasts in MSCGM had 81.82% ± 2.50% dissolved oxygen, a value significantly greater than that of both the 68.5 million chloroplast control ($p = 1.9 \times 10\text{-}5$) and the media-alone control ($p = 1.0 \times 10\text{-}6$). There was no significant difference between the dissolved oxygen concentrations of the experimental cultures with 1 million chloroplasts per mL of MSCGM and the control, MSCGM-alone cultures ($p = 0.587$). Therefore, from this experiment, it can be concluded that chloroplasts remain photosynthetically functional, viable, and sterile after being isolated and cultured for 3 days in human cell media, being able to produce large quantities of oxygen *in vitro* for a wide variety of applications.

[0038] All patents, patent applications, and published references cited herein are hereby incorporated by reference in their entirety. It should be emphasized that the above-described embodiments of the present disclosure are merely possible examples of implementations, merely set forth for a clear understanding of the principles of the disclosure. Many variations and modifications may be made to the above-described embodiment(s) without departing substantially from the spirit and principles of the disclosure. It can be appreciated that several of the above-disclosed and other features and functions, or alternatives thereof, may be desirably combined into many other different systems or applications. All such modifications and variations are intended to be included herein within the scope of this disclosure, as fall within the scope of the appended claims.

CLAIMS

We claim:

1. A method for increasing oxygen concentration in a culture media, the method comprising:

culturing chloroplasts in a culture media including one or more mammalian cells, wherein the chloroplasts remain external to the mammalian cells in the culture media; and

exposing the chloroplasts to light to cause oxygen production by the chloroplasts to enrich the culture media with oxygen to promote the growth or differentiation of the mammalian cells.

2. The method of claim 1 further comprising isolating chloroplasts from a source material.
3. The method of claim 1, wherein the mammalian cells are human stem cells.
4. The method of any one of claims 1-3, wherein the chloroplasts are provided in a concentration of about 8 million chloroplasts per ml of media and about 12 million chloroplasts per milliliter of the culture media.
5. The method of claim 1, wherein the chloroplasts are provided in a concentration of about 9 million chloroplasts per ml of media and about 11 million chloroplasts per milliliter of the culture media.
6. The method of any one of claims 1-3 or 5, wherein the chloroplasts produce between about 70 and 90% oxygen in the culture media.
7. The method of any one of claims 1-3 or 5, wherein the chloroplasts produce between about 80 and 90% oxygen in the culture media.
8. The method of any one of claim 1-2 or 4 further comprising maintaining viability of chloroplasts for at least about 72 hours.
9. The method of claim 7 further comprising maintaining viability of chloroplasts for between about 72 hours to about 120 hours.
10. A method for increasing oxygen concentration comprising:

coculturing chloroplasts with one or more mammalian cells in a media, wherein the mammalian cells under conditions that cause the one or more mammalian cells to take up the chloroplasts from the media; and

increasing oxygen production in the mammalian cells by exposing the chloroplasts to light.

11. The method of claim 10 further comprising isolating chloroplasts from a source material.

12. The method of claim 10, wherein the mammalian cells are human stem cells.
13. The method of any one of claims 10-12, wherein the chloroplasts are provided in a concentration of about 8 million chloroplasts per ml of media and about 12 million chloroplasts per milliliter of the culture media.
14. The method of claim 10, wherein the chloroplasts are provided in a concentration of about 9 million chloroplasts per ml of media and about 11 million chloroplasts per milliliter of the culture media.
15. The method of any one of claims 10-12 or 14, wherein the chloroplasts produce between about 70 and 90% oxygen in the culture media.
16. The method of any one of claims 10-12 or 14, wherein the chloroplasts produce between about 80 and 90% oxygen in the culture media.
17. The method of any one of claims 10-12 or 14 further comprising maintaining viability of chloroplasts for at least about 72 hours.
18. The method of any one of claims 10-12 or 14 further comprising maintaining viability of chloroplasts for between about 72 hours to about 120 hours.
19. A culture medium comprising chloroplasts in a concentration of about 8 million chloroplasts per ml of media and about 12 million chloroplasts per milliliter of the culture media.
20. The culture medium of claim 19, wherein the chloroplasts are provided in a concentration of about 9 million chloroplasts per ml of media and about 11 million chloroplasts per milliliter of the culture media.
21. The culture medium of claim 19, wherein the chloroplasts produce between about 70 and 90% oxygen in the culture media.
22. The culture medium of claim 19, wherein the chloroplasts produce between about 80 and 90% oxygen in the culture media.
23. The culture medium of claim 19, wherein viability of chloroplasts is maintained for at least about 72 hours.
24. The culture medium of claim 19, wherein viability of chloroplasts is maintained for for between about 72 hours to about 120 hours.
25. The culture medium of any one of claims 19-24 further comprising mammalian cells, wherein

increasing oxygen production in the mammalian cells by exposing the chloroplasts to light.

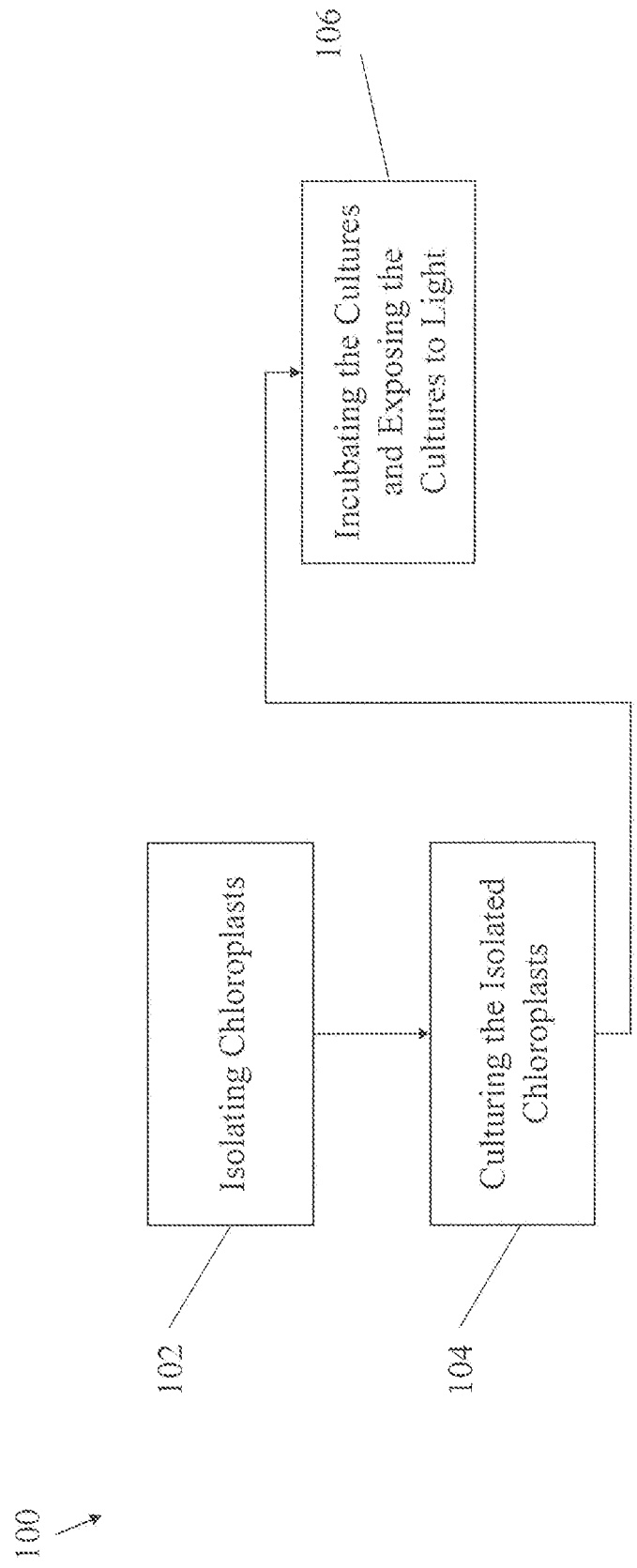
100
Isolating Chloroplasts
102
Culturing the Isolated Chloroplasts
104
Incubating the Cultures and Exposing the Cultures to Light
106

Fig. 1A

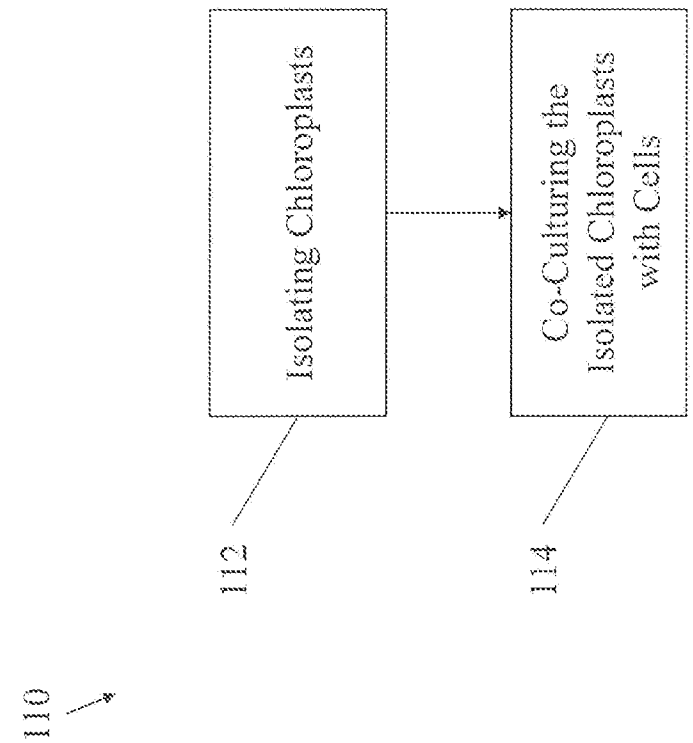
Isolating Chloroplasts
Co-Culturing the Isolated Chloroplasts with Cells
112
114
110

Fig. 1B

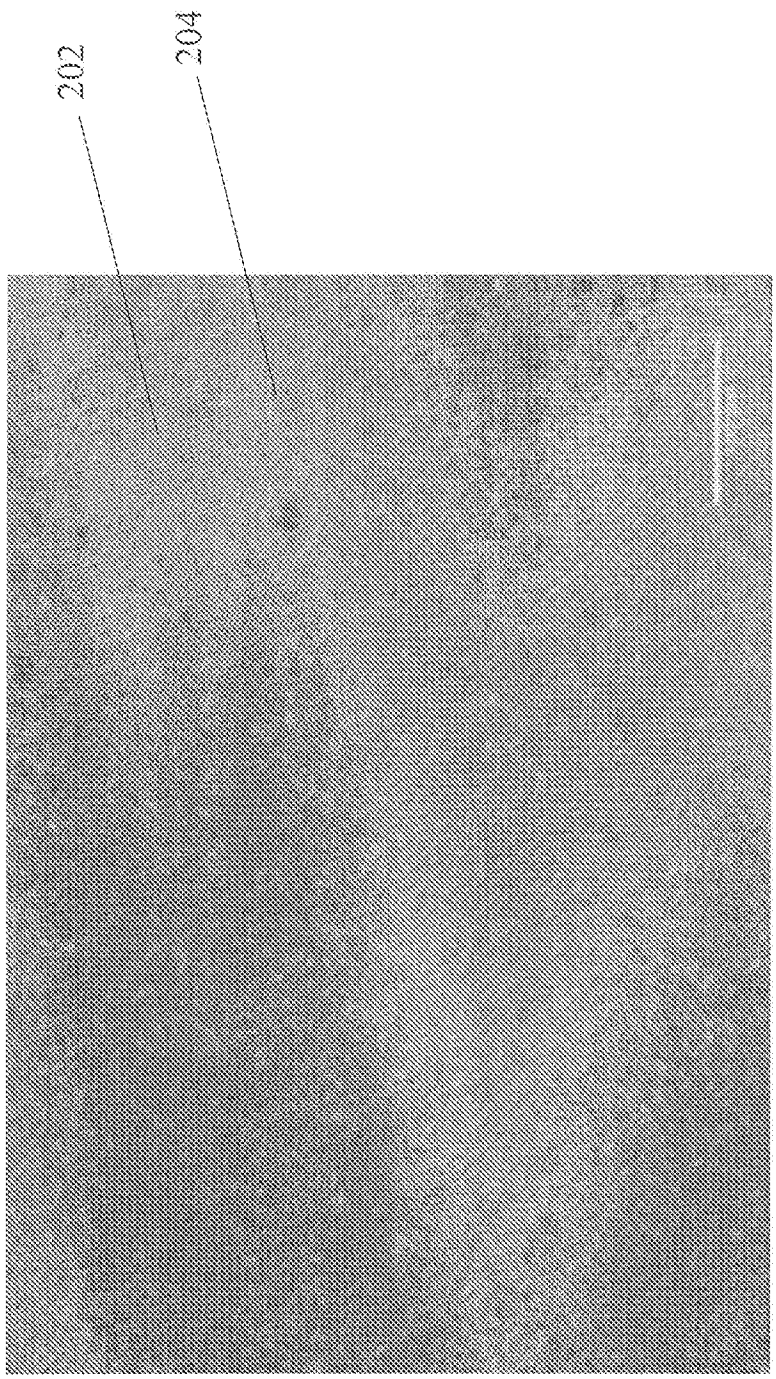

Fig. 2A

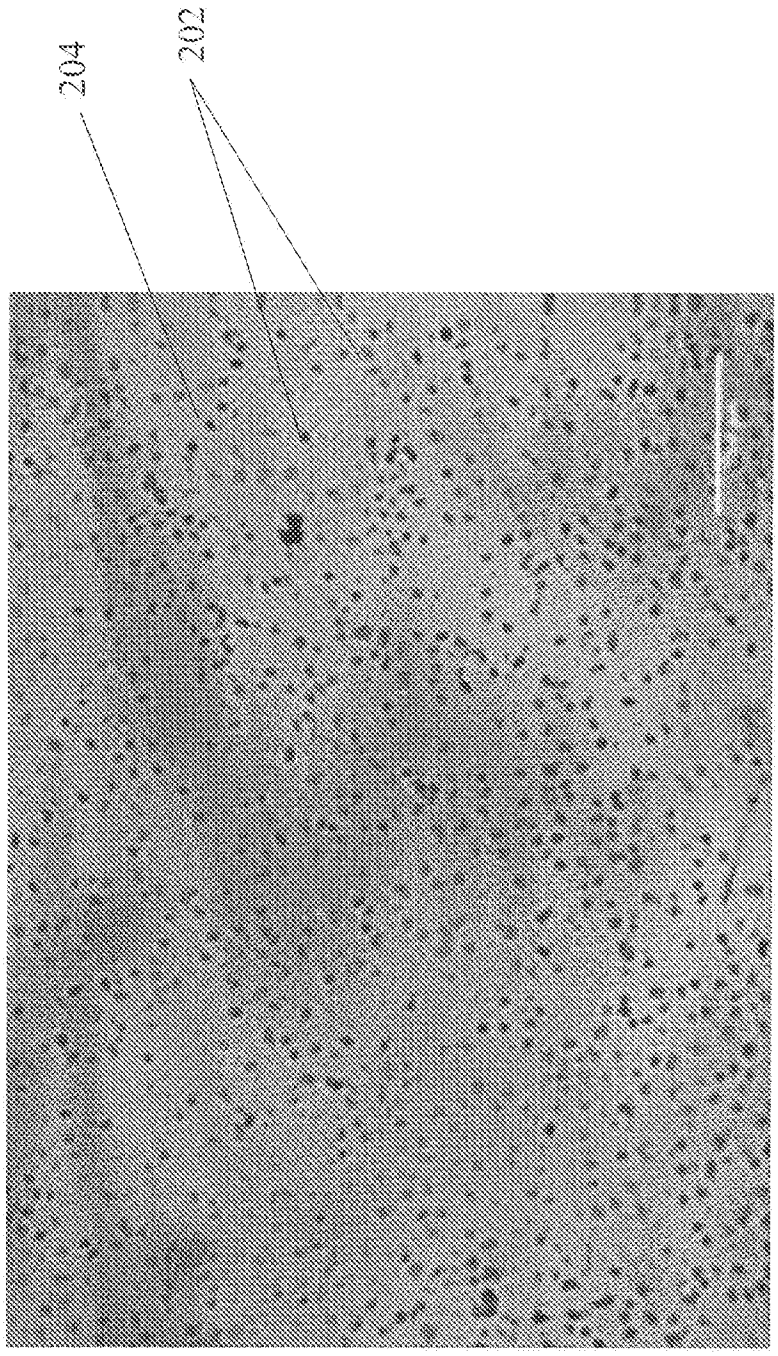

Fig. 2B

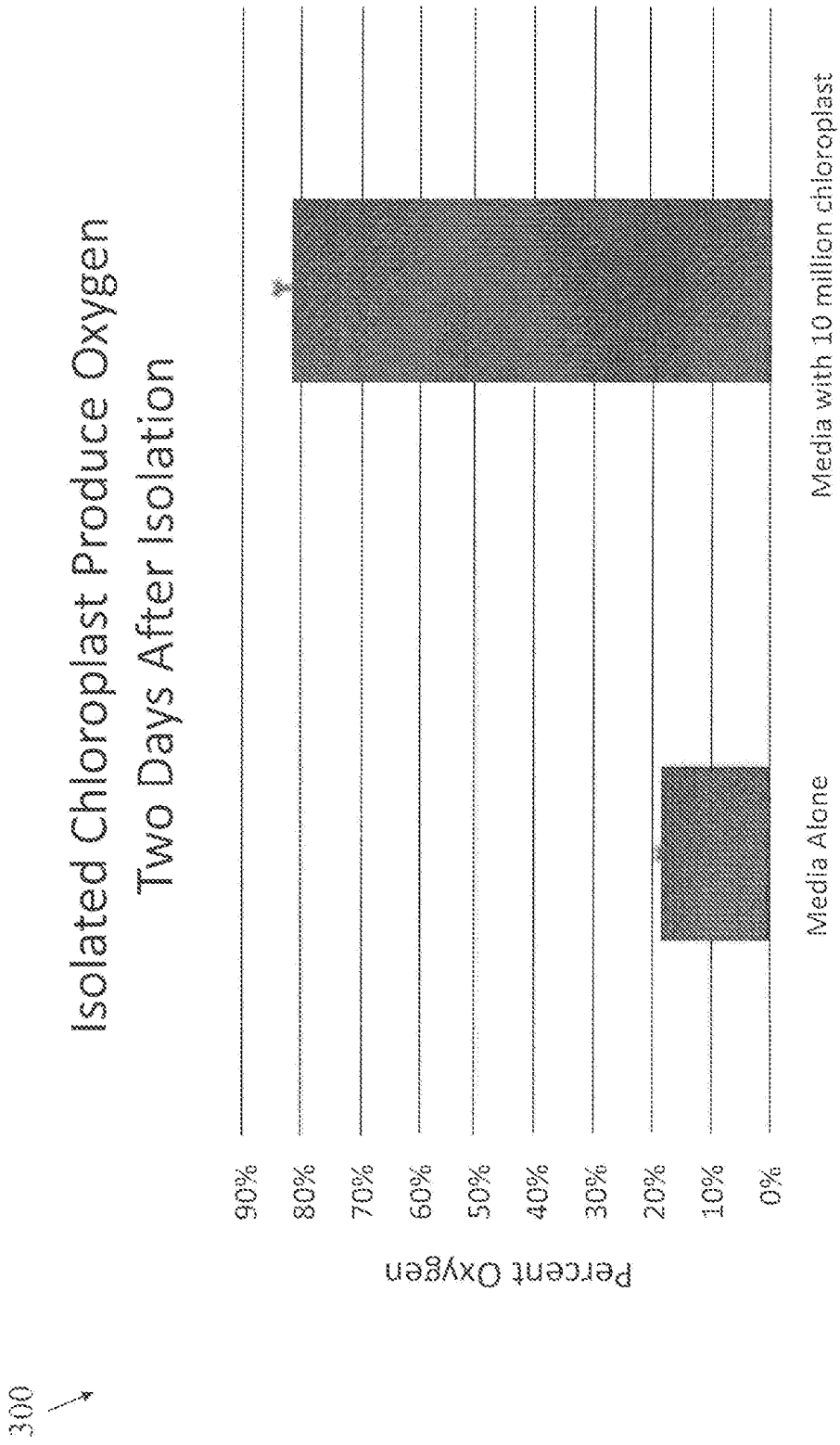
300
Isolated Chloroplast Produce Oxygen
Two Days After Isolation
Percent Oxygen
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Media Alone
Media with 10 million chloroplast

Fig. 3

Fig. 4A

Fig. 4B

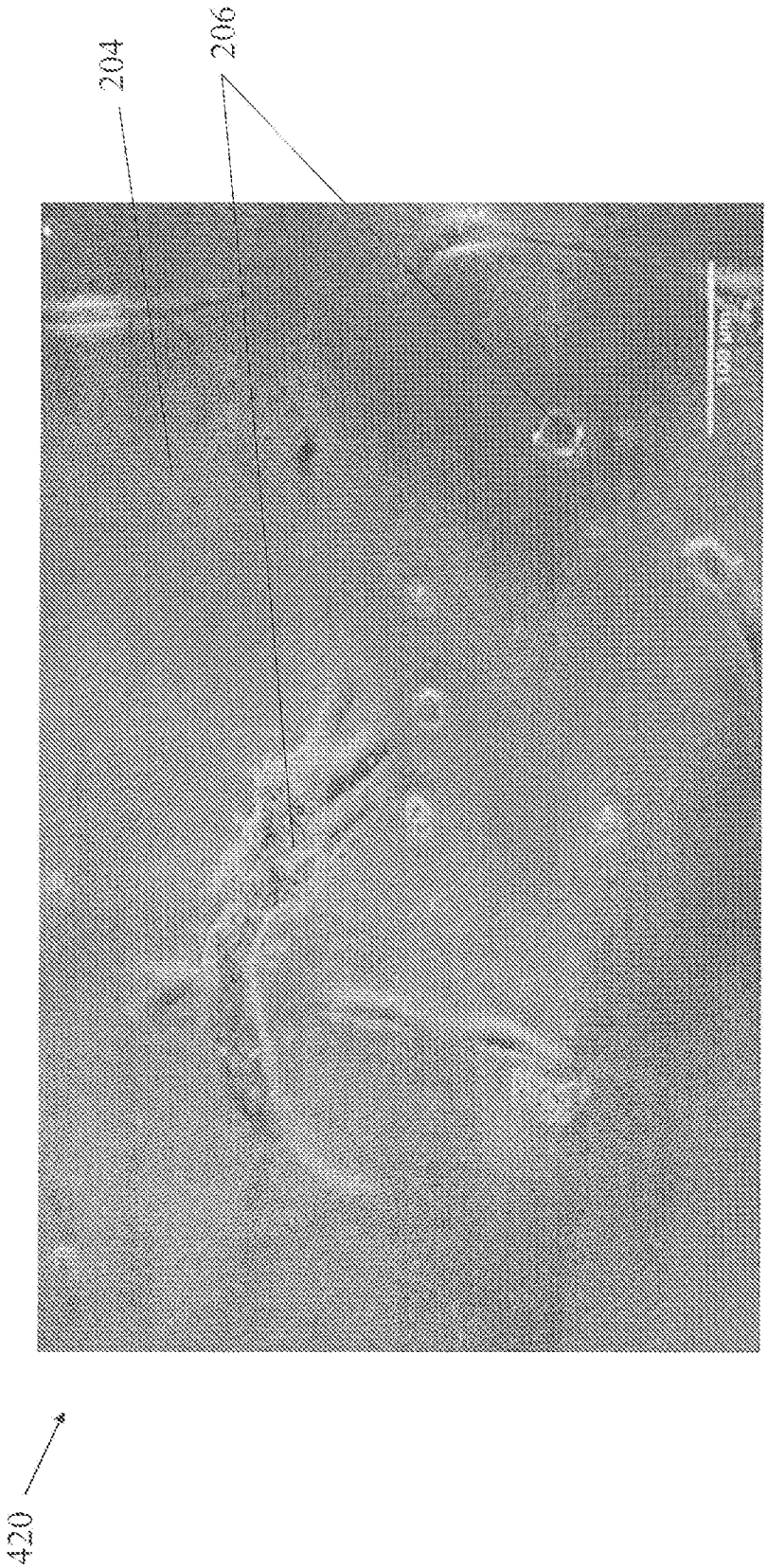

Fig. 4C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/052231

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 5/07; C12N 5/0789; C12N 5/0797 (2019.01)
CPC - C12N 5/04; C12N 5/06; C12N 5/0662; C12N 2529/10 (2019.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/93.7; 435/325; 435/375; 435/377; 435/419 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DIAMOND, I. "Can Plant Chloroplasts Function in Mammalian Cells?," Thesis, Worcester Polytechnic Institute, 26 April 2018 (26.04.2018), Pgs. 1-35. Retrieved from the Internet: <www.wpi.edu/Pubs/E-project/Available/E-project-042618-120236/unrestricted/MQP_DIAMOND_FINAL_APRIL_26_2018.pdf> on 12 November 2019 (12.11.2019). entire document	19-25
A	AGAPAKIS et al. "Towards a synthetic chloroplast," PLoS One, 20 April 2011 (20.04.2011), Vol. 6, Iss. 4, e18877, Pgs. 1-8. entire document	1-7, 9-25
A	US 2018/0110814 A1 (SYMBIOX, INC.) 26 April 2018 (26.04.2018) entire document	1-7, 9-25
A	NASS et al. "Uptake of isolated chloroplasts by mammalian cells," Science, 12 September 1969 (12.09.1969), Vol. 165, Pgs. 1128-1131. entire document	1-7, 9-25
A	SERODIO et al. "Photophysiology of kleptoplasts: photosynthetic use of light by chloroplasts living in animal cells," Philos Trans R Soc Lond B Biol Sci, 03 March 2014 (03.03.2014), Vol. 369, Pgs. 1-6. entire document	1-7, 9-25
A	US 2016/0058861 A1 (SYMBIOX, INC.) 03 March 2016 (03.03.2016) entire document	1-7, 9-25
A	US 2018/0155673 A1 (TOKYO WOMEN'S MEDICAL UNIVERSITY) 07 June 2018 (07.06.2018) entire document	1-7, 9-25

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 November 2019	12DEC 2019

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/052231

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 8
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.