

Journal of Visualized Experiments

Electrochemotherapy in 3D Ocular Melanoma Spheroids using a Customized Electrode

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60611R3
Full Title:	Electrochemotherapy in 3D Ocular Melanoma Spheroids using a Customized Electrode
Section/Category:	JoVE Medicine
Keywords:	electrochemotherapy; uveal melanoma; conjunctival melanoma; ocular melanoma; tumor spheroids; Treatment; bleomycin; electroporation; in vitro; 3D tumors
Corresponding Author:	Miltiadis Fiorentzis, MD Universitätsklinikum Essen Essen, GERMANY
Corresponding Author's Institution:	Universitätsklinikum Essen
Corresponding Author E-Mail:	miltiadis.fiorentzis@gmail.com
Order of Authors:	Miltiadis Fiorentzis, MD Arne Viestenz Berthold Seitz Sarah E Coupland Joana Heinzelmann
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US\$4,200)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	Halle (Saale)

TITLE:**Electrochemotherapy in 3D Ocular Melanoma Spheroids using a Customized Electrode****AUTHORS AND AFFILIATIONS**Miltiadis Fiorentzis^{1,2}, Arne Viestenz¹, Berthold Seitz³, Sarah E Coupland⁴, Joana Heinzelmänn¹

1. Department of Ophthalmology, University Hospital Halle (Saale), Martin-Luther University Halle-Wittenberg, Halle (Saale), Germany

2. Department of Ophthalmology, University Hospital Essen, Duisburg Essen University, Essen, Germany

3. Department of Ophthalmology, Saarland University Medical Center, Homburg/Saar, Germany

4. Liverpool Ocular Oncology Research Group, Department of Molecular and Clinical Cancer Medicine, Institute of Translational Medicine, University of Liverpool, Liverpool, United Kingdom

Corresponding Author:

Miltiadis Fiorentzis

Email: miltiadis.fiorentzis@gmail.com

Email addresses of the authors:

Miltiadis Fiorentzis: miltiadis.fiorentzis@gmail.com

Arne Viestenz: arne.viestenz@uk-halle.de

Berthold Seitz: berthold.seitz@uks.eu

Sarah E. Coupland: s.e.coupland@liverpool.ac.uk

Joana Heinzelmänn: Joana.Heinzelmänn@uk-halle.de

KEY WORDS:

electrochemotherapy; uveal melanoma; conjunctival melanoma; ocular melanoma; tumor spheroids; treatment; bleomycin; electroporation; in vitro; 3D tumors

SUMMARY:

Here, we present a protocol for the development of 3D conjunctival and uveal melanoma spheroids and the use of hand-held customized electrodes for in vitro electrochemotherapy of 3D spheroids in a culture well. This offers new perspectives in the use of electrochemotherapy in ocular melanoma treatment.

ABSTRACT

Electrochemotherapy (ECT) is the combination of transient pore formation following electric pulse application with the administration of cytotoxic drugs, which enhances the cytotoxic effect of the applied agent due to membrane changes. In vitro 3D culture systems simulate the in vivo tumor growth and preserve the biological characteristics of tumors more accurately than conventional monolayer cell cultures. We describe a protocol for the development of 3D tumor organoids using conjunctival melanoma (CM) and uveal melanoma (UM) cell lines as well as the use of hand-held customized electrodes, suitable for in vitro ECT in the culture well without

destruction of the tumor environment. This protocol analyzes the culture and growth of 3D CM and UM spheroids and their reaction to bleomycin (2.5 µg/mL) alone, electroporation (EP) (750 Volts/cm, 8 pulses, 100 µs, 5 Hz) alone, and ECT as a combination of EP and bleomycin. The drug concentration and the EP settings used in this protocol were established as preferred ECT conditions according to previous experiments. The assay used to determine the spheroid viability was conducted 3-7 days following treatment. The effect on viability and growth of the 3D tumor spheroids was significant only after ECT. The customized electrodes are described in detail in order to facilitate the application of pulses in the culture well. This novel treatment of 3D UM and CM spheroids sets a steppingstone for future clinical application.

INTRODUCTION

Uveal melanoma (UM) is the most common primary intraocular tumor in adults, whereas conjunctival melanoma (CM) represents 2% of all ocular melanomas¹⁻⁶. Brachytherapy, proton beam radiotherapy, and phototherapy are the first line treatments in UM, whereas enucleation of the globe may be necessary¹⁻³. The treatment of CM varies between ocular oncology centers; excisional biopsy followed by local chemotherapy and/or radiation therapy is the most frequent treatment approach⁴. Despite treatment, CM is associated with a mortality of 25%–30%⁵.

There is a paucity of literature on the formation of CM and UM spheroids and the application of ECT in ocular melanoma⁶⁻⁸. Tumor spheroids have better biological features than conventional 2D cell cultures and have been proposed as a useful tool to mimic the in vivo tumor environment⁹. Electrochemotherapy (ECT) combines the use of non-permeable cytotoxic drugs with electroporation (EP)¹⁰. EP is the local application of short and intense electric pulses that transiently permeabilizes cells for a localized increase of anti-cancer drug uptake in cancer cells and results in increased cell death¹¹. This study establishes a protocol describing the development of CM and UM spheroids and investigates the results following ECT with bleomycin. This protocol could assist investigators in the field of ocular oncology using other therapeutic modalities on spheroids or study further effects of ECT. Due to the limited use of ECT in ophthalmology, there is little knowledge regarding the effect and process of this modality; thus, this experiment may widen the spectrum of treatment options in the future. We propose a novel customized hand-held plate electrode setting, which allows the ECT of the spheroid in the culture wells without any destruction of the tumor environment.

PROTOCOL

1. Spheroid formation

1.1. Use adherently growing cancer cell lines for spheroid formation.

1.2. Provide all cell culture associated steps under sterile bench conditions.

1.3. Prepare the standard complete culture medium as recommended for the cell lines of interest and warm to 37 °C using a water bath.

NOTE: Here, the human conjunctival cell lines CM2005.1, CRMM1, CRMM2 as well as the uveal melanoma cell lines 92.1, OMM1 and OMM2 were used and cultured in Ham F-12 medium containing 10% fetal bovine serum and RPMI containing 10% fetal bovine serum, respectively.

1.4. Detach the cells of interest using standard enzymatic digestion methods (e.g., trypsin-EDTA solution).

1.5. To determine the number of vital cells, resuspend 10 μ L of the cell suspension in 10 μ L of Trypan blue solution (1:1) and count the cells using a chamber (hemocytometer or an automated cell counter) within 5 min. Cells should be <90% of confluence and in good condition.

1.6. To form spheroids, seed 5×10^3 cells per well with a total volume of 200 μ L of complete culture medium in 96-well ultra-low attachment plates. Incubate the plate at 37 °C in a humidified atmosphere containing 5% CO₂.

NOTE: For effective inhibition of cellular attachment, use ultra-low attachment plates with a covalently bound hydrophilic, non-ionic, neutrally charged hydrogel on the surface. This hydrogel inhibits immortalization of cells and force them into a suspended state to build 3D spheroids.

1.7. Check the spheroid formation using a microscope.

NOTE Some cell lines do not form round 3D tumor spheroids in the first passage. Passage the tumor sphere culture before they start to develop a dark center. Dissociate the tumor masses depending on the cell line after 4-10 days by trypsinization. Afterwards, seed the single cells in a fresh 96 ultra-low attachment plate. By repeating this procedure for several passages, cells will adapt to 3D culture.

2. Electrochemotherapy of the tumor spheroids

2.1. As an assay control, use untreated spheroids and spheroids treated only with EP or bleomycin alone.

2.2. Adjust the settings for the electroporator (pulse counts, pulse frequency, duration time and voltage; see Table of Materials). For melanoma cells, use 8 square wave electric pulses of 750 Volts/cm strength, 100 μ s pulse duration, 5 Hz repetition frequency.

2.3. At day 3 of spheroid culture, prepare fresh bleomycin sulfate solution in sterile PBS at a concentration of 5 μ g/mL in a 15 mL tube.

2.4. Exchange the cell culture medium of the spheroids by removing 200 μ L of culture medium and refilling with 100 μ L of fresh complete culture medium for each well.

2.5. Add 100 μ L of bleomycin sulfate solution to each well (final concentration 2.5 μ g/mL). Add 100 μ L of fresh medium to untreated controls and spheroids only treated with EP instead of bleomycin sulfate solution.

2.6. Perform the electroporation.

NOTE: The electrodes shown in Figure 1 were produced in the research workshop of the University of Halle-Wittenberg. They are made of stainless steel and fit the grip of the electroporator. The electrodes were designed for use in 96 well ultra-low attachment plates. Similar stainless-steel electrodes can be easily manufactured for alternative applications too.

2.6.1. Sterilize electrodes with 70% ethanol and allow electrodes to dry. The diameter of the electrodes is 1 mm, the gap between the two electrodes is 4 mm, and the length of each electrode is 8 mm (Figure 1a).

2.6.2. Place the electrodes at the bottom of the well, with the spheroids between the electrodes. This allows the ideal positioning of the spheroid between the two electrodes (Figure 1b).

2.6.3. Shake the plate to allow the spheroids to move from the bottom of the well and be placed between the electrodes. The spheroid will only be between the electrodes for a couple of seconds.

2.6.4. Start the electroporation with preferred settings.

2.6.5. Replace the bleomycin sulfate solution in the well by exchanging 150 μ L of medium with 150 μ L of fresh medium.

2.6.6. Incubate the cells for up to 10 days at 37 °C in a humidified atmosphere containing 5% CO₂. If the untreated spheroids start to develop a dark center, terminate the experiment. Exchange 150 μ L of the cell culture medium every 2-3 days. Thereby, avoid spheroid disruption by positioning the pipette tip at the edge region of the well, tilting the plate and slowly pipetting.

3. Determination of spheroid size

3.1. Measure the spheroid size on the day of treatment (starting point) and 3-7 days following treatment using bright field microscopy by calculating the cross sectional area of the spheroids using ImageJ Fiji¹¹.

3.2. Take an image of the single spheroids with the scale bar. Use a magnification by which the whole spheroid is visible. For example, 5x magnification was used for CM2005.1. Save the image as a JPEG.

3.3. Analyze the spheroid size by calculating the cross-sectional area using ImageJ/Fiji.

3.3.1. Install the software (<https://imagej.net/Fiji/Downloads>).

3.3.2. Start ImageJ/Fiji. Import the spheroid-image: **File > Open**.

3.3.3. To set the scale, choose the line button of the toolbar and mark the line of the scale bar in the image by mouse click: **Analyze > Set Scale**. Using a known distance, fill in the distance of the scale bar. Change the Unit of length to μm . Click the field global to assign the settings for all images with the same magnification factor.

3.3.4. Convert the image to 8-bit: **Image > Type > 8-bit**.

3.3.5. Choose **Image > Adjust > Auto Threshold (Select > Do not reset range)**. The spheroid is marked in red or black depending on the threshold marker chosen. Adjust the sliders so that the spheroid pixels turn red, but non-spheroid pixels do not change color. Close the Threshold dialog window without clicking any of the buttons.

3.3.6. To calculate the cross-sectional area of the spheroid, choose **Analyze > Set Measurements** and click **Area**, **Limit to Threshold** and **Display label**. Close the **Set Measurements** dialog window by clicking **OK**.

3.3.7. To show the results, choose **Analyze > Analyze Particles** and change the size (μm^2) = 10000-infinity to remove noise. Click the button **Display results** and close the window by clicking **OK**. In the **Results**, the spheroids with the label and area are shown. By repeating the analyses of several spheroids, the results are listed in the **Results** window.

3.3.8. To determine the relative treatment response of the ECT treated spheroids compared to the untreated and single treated controls, calculate the percentage changes of the cross-sectional area.

4. Determination of spheroid viability

4.1. Measure the spheroid viability 3-7 days following treatment using a cell viability assay suitable for 3D cell cultures according to the manufacturer instructions. The assay needs to penetrate and lyse large spheroids to allow detection of viability by quantitation of ATP, which signals the presence of metabolically active cells.

NOTE: In this experiment, a luminescence-based assay as a readout method was used. The viability of the ECT-treated spheroids was compared to controls seven days following treatment.

REPRESENTATIVE RESULTS

The experiments were conducted with customized hand-held electrodes, which are composed of high-grade stainless steel. The thickness of the electrodes is 1 mm, the width is 4 mm, the gap between the two electrodes is 4 mm and the length of each electrode is 8 mm (**Figure 1**). EP and bleomycin alone have no significant effect on the viability and growth of both UM and CM tumor spheroids. ECT shows a significant reduction in tumor viability and in spheroid size. Loss of spheroid architecture with deconstructed cell fragments around the spheroids and necrosis on the central and peripheral region of all tested spheroids were observed following ECT with bleomycin. **Figure 2** shows the results of the CM2005.1 cell lines. Metastatic UM cell lines showed a higher response compared to primary cell lines following ECT⁸.

FIGURE LEGENDS

Figure 1: Customized hand-held electrodes. The electrodes are made of high-grade stainless steel. The handle of the electrodes is provided with the electroporator. The thickness of the electrodes is 1 mm, the width is 4 mm, the gap between the two electrodes is 4 mm and the length of each electrode is 8 mm (a); electroporation of spheroids in a 96 well format (b).

Figure 2: Cytotoxic effect of electrochemotherapy on tumor spheroids of the conjunctival melanoma cell line CM2005.1. Electrochemotherapy (ECT, 750 V/cm after application of 2.5 µg/mL bleomycin) caused stronger cytotoxic effects in spheroids compared to electroporation (EP) alone or chemotherapy using bleomycin (2.5 g/mL) alone. The cytotoxic effect was measured by calculating both the cross-sectional area and a viability assay as a percentage of the untreated control seven days after treatment. Box plots show the mean cross-sectional area of spheroids (a); the mean viability of spheroids (b); representative images of spheroids, scale bar = 200 µm (c).

DISCUSSION

EP is used in various biotechnological and clinical applications¹². New technological developments, such as specially designed electrodes with high specificity for every target cell and site, may help ECT target tissue anywhere in the body¹². The design and position of the electrodes must allow complete tumor accessibility and ensure that healthy tissue is only minimally affected or not damaged from the treatment¹³.

Previous publications showed the effect of ECT in human melanoma cell suspensions in vitro^{7,8}. The literature referring to the application of ECT in 3D ocular cell models or other similar in vivo environments, enabling a safer therapeutic utilization, is limited. Brun et al. postulate that the 3D cells in the scaffold during the morphological analysis have a round shape different from the elongated shape shown in the 2D cultures but extremely similar to the cells from biopsies of patients⁹. The refinement of therapy settings and instruments used in 3D cultures may lead to an optimization of ECT parameters, allowing a more accurate clinical approach⁹.

We describe a technological development regarding new electrodes for the application of ECT in 3D cell cultures. Bleomycin is the most commonly administered cytotoxic agent in combination with ECT¹¹. Previous studies of our group showed that the applied EP settings (750 Volts/cm, 8 pulses, 100 ms, 5 Hz) were suitable for treatment of ocular tumors in vitro. Critical steps of the

technique include the short time needed to perform the ECT while the spheroid is sinking after mobilization as well as the precise dimension of the electrodes. The necessity for the customized electrodes was due to difficulties in performing ECT in the wells with the available instruments. Unpublished data from our group showed increased spheroid damage when transferring the spheroids into a bigger well or into a cuvette to prepare them for treatment and then back to the culture well. An advantage of the described technique is the lack of spheroid manipulation to perform the treatment, because the organoids are not transferred into bigger plates or wells. Therefore, all spheroids retain their form. Another advantage is the use of more robust 3D viability assays to determine cytotoxicity of ECT compared to standard viability assays, such as the MTT assay. Thereby, all cells of the spheroid are lysed and additional cell washing, removal of medium and multiple pipetting steps are required.

Limitations of the described methods are the short lifespan of spheroids, which affects the size of the tumor as well as the cell necrosis, as seen in the center of the tumor organoid. The associated high mortality rate in both CM and UM and the limited therapeutic options require the enrichment of the existing therapeutic possibilities. ECT may offer an adjuvant modality for the improvement of the patient's quality of life and prolong the patient's survival. These in vitro conditions imitate an in vivo setting with higher precision, offering promising results for further human application. Future studies using spheroids prepared from primary cultures can deliver more representative results for optimization of ECT settings for a targeted treatment.

ACKNOWLEDGEMENTS

This study was supported by Dr. Rolf M. Schwiete-Stiftung. The authors thank Martine J. Jager (Laboratory of Ophthalmology at LUMC, Leiden, The Netherlands) and Helen Kalirai (Liverpool Ocular Oncology Research Group, Molecular and Clinical Cancer Medicine, University of Liverpool, UK) for providing the UM cell lines. We would also like to thank Sabine Hecht (Department of Ophthalmology, University Hospital Halle (Saale), Germany) for technical assistance.

DISCLOSURES

This report received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

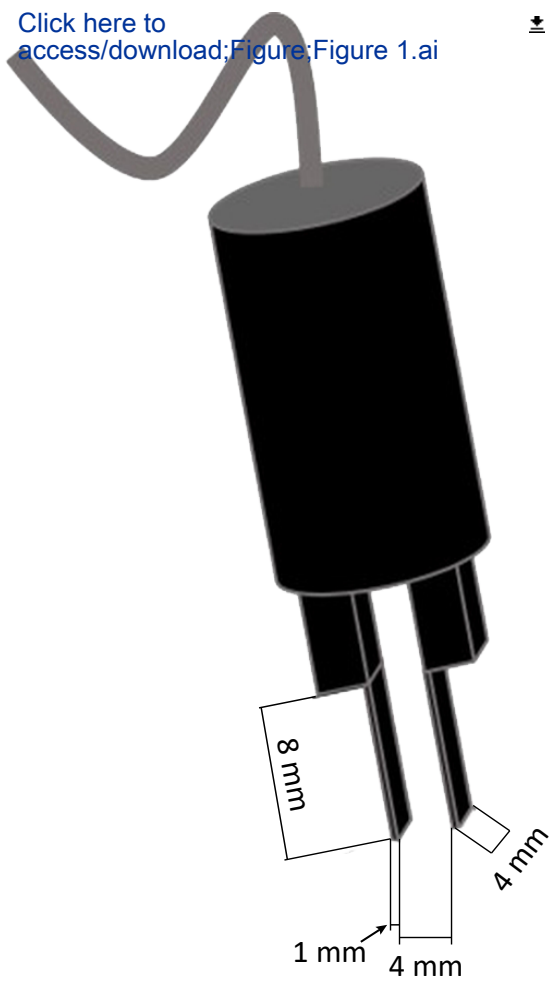
REFERENCES

1. Kaliki, S., Shields, C.L. Uveal melanoma: relatively rare but deadly cancer. *Eye (Lond)*. **31** (2), 241-257 (2017).
2. Chang, A.E., Karnell, L.H., Menck, H.R. The National Cancer Data Base report on cutaneous and noncutaneous melanoma: a summary of 84,836 cases from the past decade. *The American College of Surgeons Commission on Cancer and the American Cancer Society. Cancer*. **83** (8), 1664-78 (1998).
3. Dogrusoz, M., Jager, M.J., Damato, B. Uveal Melanoma Treatment and Prognostication. *Asia-Pacific Journal of Ophthalmology*. **6** (2), 186-196 (2017).
4. Damato, B., Coupland, S.E. Management of conjunctival melanoma. *Expert Review of Anticancer Therapy*. **9** (9), 1227-39 (2009).

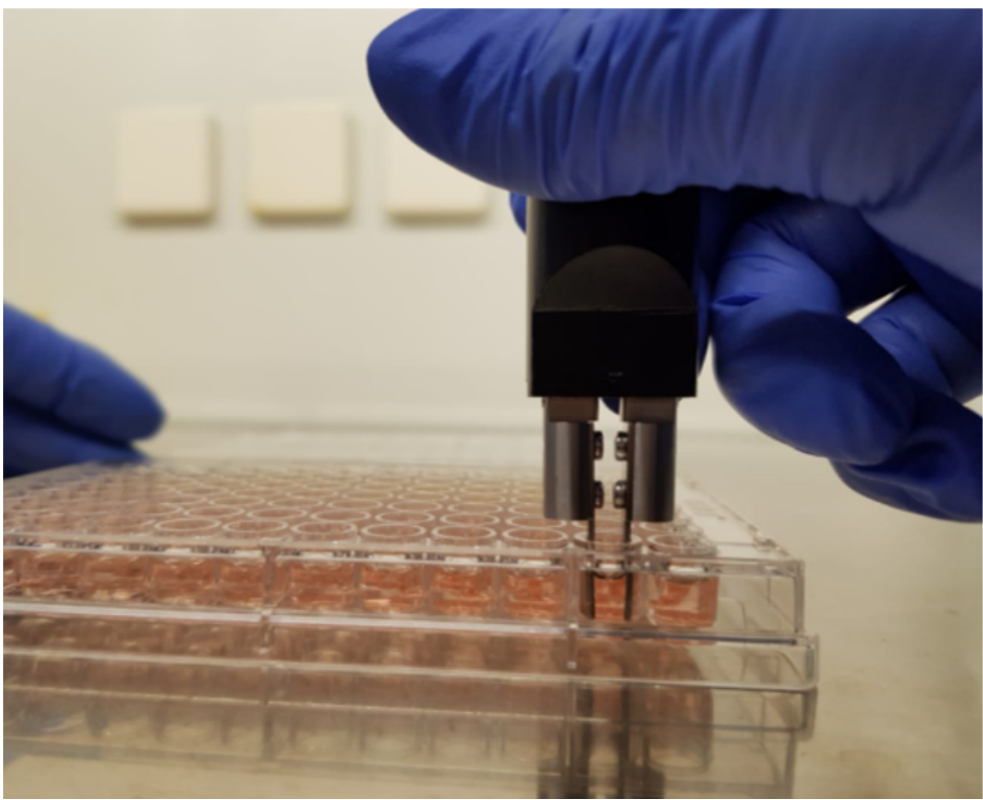
5. Norregaard, J.C., Gerner, N., Jensen, O.A., Prause, J.U. Malignant melanoma of the conjunctiva: occurrence and survival following surgery and radiotherapy in a Danish population. *Graefe's Archive for Clinical and Experimental Ophthalmology*. **234** (9), 569-72 (1996).
6. Fiorentzis, M. et al. Electrochemotherapy with bleomycin and cisplatin enhances cytotoxicity in primary and metastatic uveal melanoma cell lines in vitro. *Neoplasma*. **65** (2), 210-215 (2018).
7. Fiorentzis, M. et al. Conjunctival melanoma and electrochemotherapy: preliminary results using 2D and 3D cell culture models in vitro. *Acta Ophthalmologica*. **97** (4), e632-e640 (2019).
8. Fiorentzis, M. et al. The Potential Use of Electrochemotherapy in the Treatment of Uveal Melanoma: In vitro Results in 3D Tumor Cultures and In vivo Results in a Chick Embryo Model. *Cancers (Basel)*. **11** (9) (2019).
9. Brun, P. et al. Cell-seeded 3D scaffolds as in vitro models for electroporation. *Bioelectrochemistry*. **125**, 15-24 (2019).
10. Sersa, G. et al. Electrochemotherapy in treatment of tumours. *European Journal of Surgical Oncology*. **34** (2), 232-40 (2008).
11. Rueden, C.T. et al. ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics*. **18** (1), 529 (2017).
12. Miklavcic, D. et al. Electrochemotherapy: technological advancements for efficient electroporation-based treatment of internal tumors. *Medical & Biological Engineering & Computing*. **50** (12), 1213-25 (2012).
13. Miklavcic, D., Corovic, S., Pucihar, G., Pavselj, N. Importance of tumour coverage by sufficiently high local electric field for effective electrochemotherapy. *EJC Supplements*. **4** (11), 45-51 (2006).

Figure 1

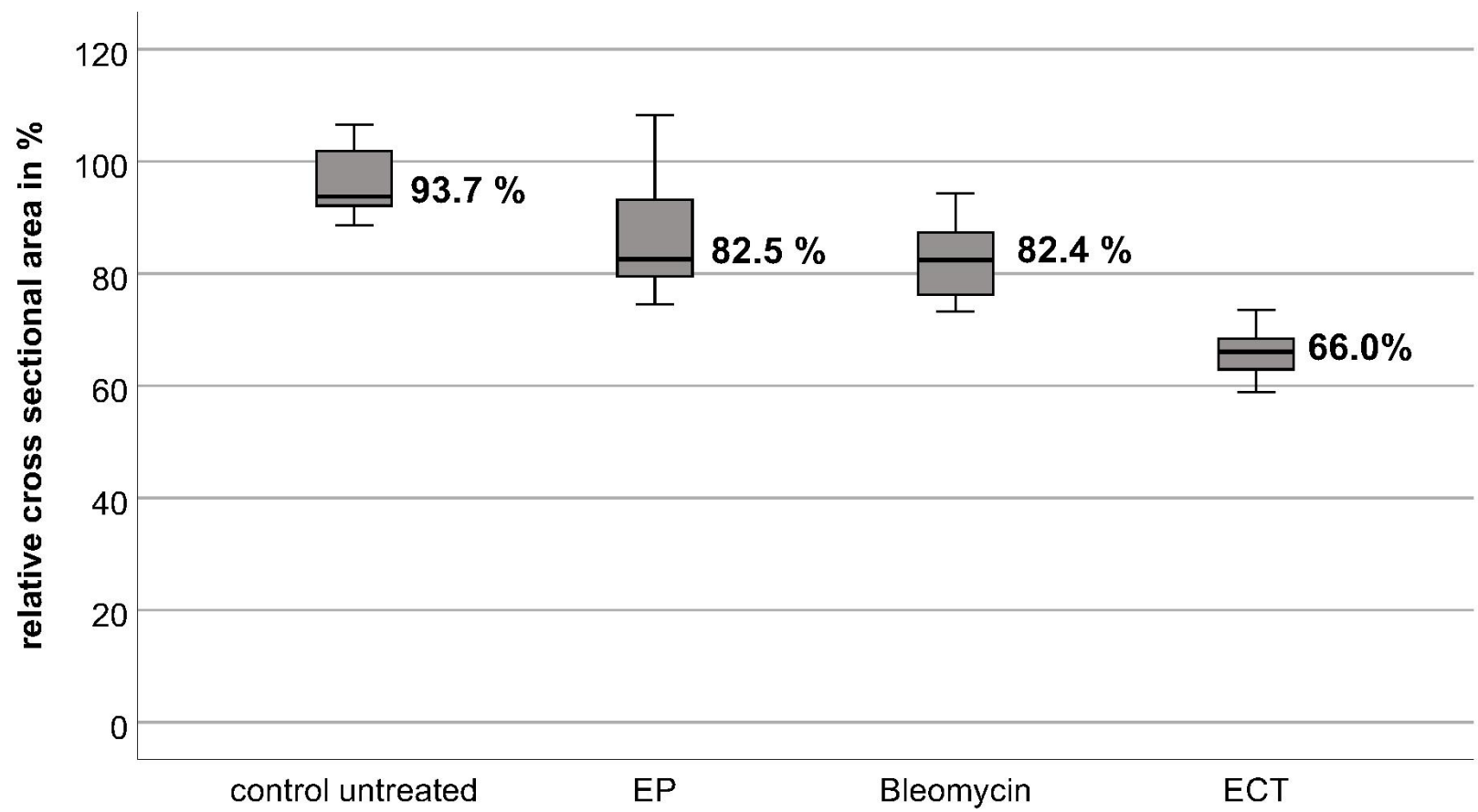
a



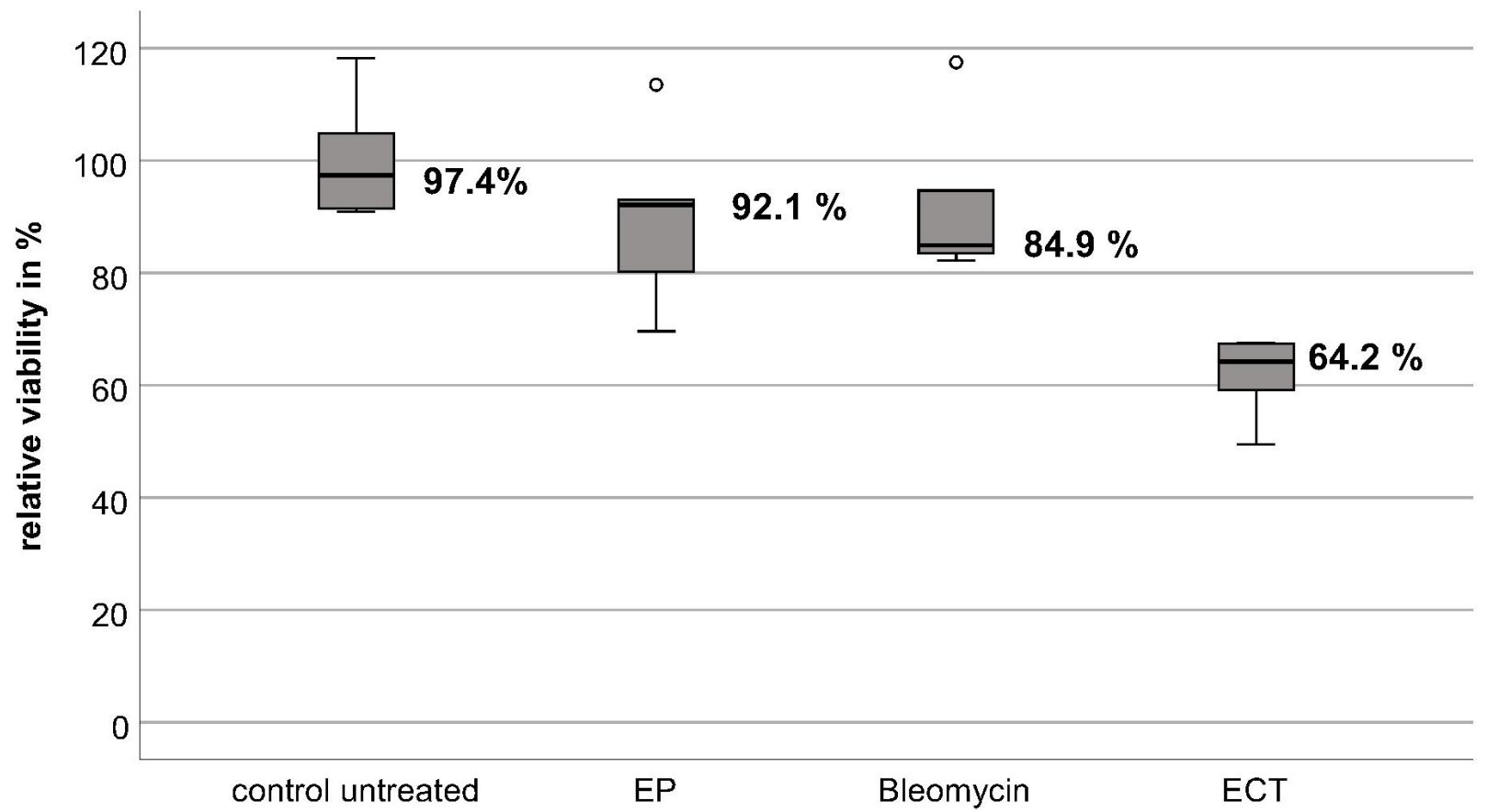
b



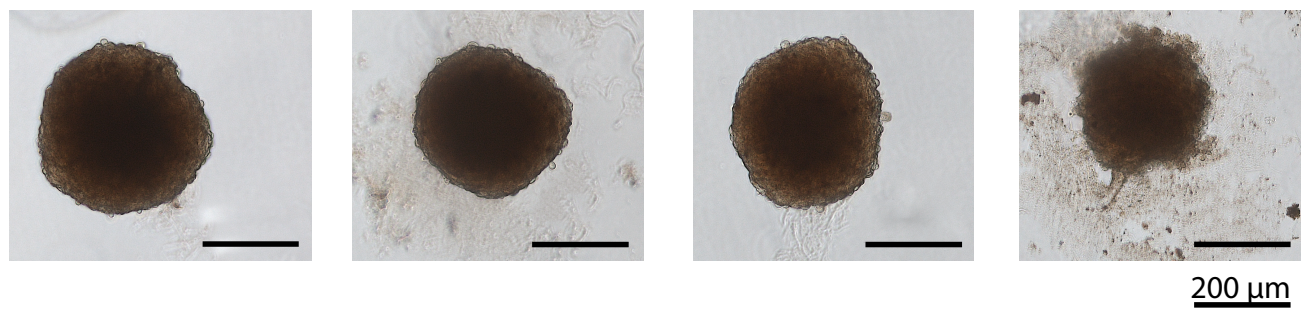
a



b



c



Name of Material/Equipment	Company	Catalog Number	Comments/Description
Bleomycin sulfate	Enzo	BML-AP302-0010	10 mg
Cell culture flask 75 cm	TPP	TPP90076	
Cell culture flask TC175 cm	Sarstedt	833,912,002	Standard with Filter
Cliniporator	IGEA	EPS01	Electroporator
Corning Costar Ultra-Low Attachment Multiple Well Plate, 96 well, round bottom	Corning	7007	96Well clear round Bottom, Ultra low attachment
EGF 1MG	Sigma-Aldrich	E4127-.1MG	
F-12 K Nut Mix (1x)	Gibco	21127-022	
Fetal Bovine Serum, South American FKS	Corning	35-079-CV	LOT Nr. 35079010, Reservierungsnummer: EU0041
KGM-2	Lonza	CC-3103	
PBS w/o. CaCl und MgCl	PromoCell	C-40232	500 ml
Penicillin-Streptomycin	Sigma-Aldrich	P4333-100ml	
RPMI 1640	Gibco	11504566	ATCC modification, HEPES, liquid, 500 ml
Tecan Infinity M Plex	Tecan		
Trypsin-EDTA 1x Solution	Sigma-Aldrich	59430C-100ml	

plates

78541



1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Author(s):

Electrochemotherapy of ocular melanoma cell-seeded 3D spheroid
Fiorenza U., Vietenz A., Seitz B., Copeland SC., Heintzelmann J.

Item 1: The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via:

☐

Standard Access

☒

Open Access

Item 2: Please select one of the following items:

☒

The Author is **NOT** a United States government employee.

☐

The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐

The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: “**Agreement**” means this Article and Video License Agreement; “**Article**” means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; “**Author**” means the author who is a signatory to this Agreement; “**Collective Work**” means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; “**CRC License**” means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; “**Derivative Work**” means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; “**Institution**” means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; “**JoVE**” means MyJoVE Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; “**Materials**” means the Article and / or the Video; “**Parties**” means the Author and JoVE; “**Video**” means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion

of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the “Open Access” box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum

rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to

the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:


Department:

Institution:

Title:

Signature:

Date:

Name:	Miltiadis Fiorentzis, MD, FRCO, FICO
Department:	Department of Ophthalmology, University
Institution:	Hospital Halle (Saale), Martin-Luther-University
Title:	Halle-Wittenberg, Halle (Saale), Germany
Signature:	
Date:	29.7.2019

Please submit a **signed and dated copy** of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

REBUTTAL LETTER FOR SUBMISSION OF MANUSCRIPT

Dr Miltiadis Fiorentzis, MD, FEBO, FICO
Department of Ophthalmology,
University Hospital Halle (Saale),
Martin-Luther University Halle-Wittenberg,
06120 Halle (Saale), Germany
Email: Miltiadis.Fiorentzis@gmail.com
Tel: +49 (0) 345-557 1878

30.10.2019

Dear Editor,

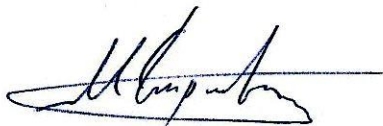
We wish to submit a revised manuscript entitled “Electrochemotherapy of ocular melanoma cell-seeded 3D spheroids” for consideration by the JoVE. Thank you very much for your comments and recommendations. We worked on all the suggested points and revised the manuscript accordingly.

We confirm that this work is original and has not been published elsewhere nor is it currently under consideration for publication elsewhere. All authors of this paper have read and approved the final version submitted.

Conflict of interest: There is no conflict of interest. I confirm that no connections exist with a company, whose product is mentioned in the article or sells a competing product. The presentation of the topic is neutral independent of the contents of the product.

Please address all correspondence concerning this manuscript to me at the address above.

Thank you for your consideration of this manuscript.



Sincerely,

Miltiadis Fiorentzis, MD, FEBO, FICO

Answer to the reviewers

Dr Miltiadis Fiorentzis, MD, FEBO, FICO
Department of Ophthalmology,
University Hospital Essen,
Duisburg Essen University,
Hufelandstraße 55
45147 Essen
Telephone: +49 201 723 2900

02.01.2020

Dear Dr Steindel,

Thank you for your email and the reviewers' comments. We carefully proofread the manuscript once more and corrected some spelling errors.

Thank you once more for your help,

M. Fiorentzis

Editorial comments:

No editorial concerns, although proofreading is always welcome.

Answer: Thank you for the positive review. We proofread the manuscript once more.

Reviewers' comments:

Reviewer #2:

Manuscript Summary:

In this manuscript, the authors describe their protocol for electrochemotherapy of ocular melanoma spheroids using their customized electrodes directly in the wells where spheroids are cultured.

In the revised version, the authors followed most of my suggestions and corrections. The title and abstract were changed significantly. The authors also added the protocol and changed many segment in the introduction and discussion. Therefore, I believe that the manuscript is now suitable for publication

Answer: Thank you for the positive review. We proofread the manuscript once more.

Reviewer #3:

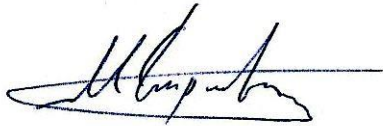
Manuscript Summary:

The revised manuscript now reflects the work conducted by the authors and includes a detailed and accurate description of the protocol followed to develop the method for electrochemotherapy of cancer spheroids. All previous questions and suggestions have been addressed and the authors have significantly improved the content of the manuscript. There are no major concerns. The authors should perform a final review of the manuscript to ensure the correction of any language or grammar issues.

Answer: Thank you for the positive review. We proofread the manuscript once more.

Please address all correspondence concerning this manuscript to me at the address above.

Thank you for your consideration of this manuscript.

A handwritten signature in blue ink, appearing to read 'M. Fiorentzis', written over a horizontal line.

Sincerely,

Miltiadis Fiorentzis, MD, FEBO, FICO



1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

Title of Article:

Author(s):

Electrochemotherapy in 3D ocular melanoma spheroids:
Fiorentino N, Viestenz A, Seitz B, Cooper SE, Hentzelman

Item 1: The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via:

☐

Standard Access

☒

Open Access

Item 2: Please select one of the following items:

☒

The Author is **NOT** a United States government employee.

☐

The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐

The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

Optimized protocol
using a customized
electrode

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution 3.0 Agreement (also known as CC-BY), the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by/3.0/us/legalcode>; "**CRC NonCommercial License**" means the Creative Commons Attribution-NonCommercial 3.0 Agreement (also known as CC-BY-NC), the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc/3.0/legalcode>; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJoVE Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its

affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License. If the "Standard Access" box

612542.6 For questions, please contact us at submissions@jove.com or +1.617.945.9051.

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC NonCommercial License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video - Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video - Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with

such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or

decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:

Department:

Institution:

Title:

Signature:

Date:

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140