Journal of Visualized Experiments

Circulating tumor cell lines: an innovative tool for fundamental and translational research

--Manuscript Draft--

Article Type:	Methods Article - Author Produced Video
Manuscript Number:	JoVE62329R5
Full Title:	Circulating tumor cell lines: an innovative tool for fundamental and translational research
Corresponding Author:	Julie Pannequin IGF: Institut de genomique fonctionnelle FRANCE
Corresponding Author's Institution:	IGF: Institut de genomique fonctionnelle
Corresponding Author E-Mail:	julie.pannequin@igf.cnrs.fr
Order of Authors:	Guillaume Belthier
	Zeinab Homayed
	Céline Bouclier
	Muriel Asari
	Julie Pannequin
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please specify the section of the submitted manuscript.	Cancer Research
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release
Please provide any comments to the journal here.	

TITLE:

Circulating Tumor Cell Lines: an Innovative Tool for Fundamental and Translational Research

AUTHORS AND AFFILIATIONS:

Guillaume Belthier*
Montpellier University
Institute for Functional Genomics
Department of Physiology and Cancer
UMR5203 CNRS
U1191 INSERM
Montpellier, France
guillaume.belthier@igf.cnrs.fr

Zeinab Homayed*
Montpellier University
Institute for Functional Genomics
Department of Physiology and Cancer
UMR5203 CNRS
U1191 INSERM
Montpellier, France
zeinab.homayed@igf.cnrs.fr

*These authors have equally contributed

Céline Bouclier
Montpellier University
Institute for Functional Genomics
Department of Physiology and Cancer
UMR5203 CNRS
U1191 INSERM
Montpellier, France
celine.bouclier@igf.cnrs.fr

Muriel Asari
Montpellier University
Institute for Functional Genomics
UMR5203 CNRS
U1191 INSERM
Montpellier, France
muriel.asari@igf.cnrs.fr

Julie Pannequin
Montpellier University
Institute for Functional Genomics
Department of Physiology and Cancer
UMR5203 CNRS
U1191 INSERM

Montpellier, France
julie.pannequin@igf.cnrs.fr

Corresponding Author: Julie Pannequin
julie.pannequin@igf.cnrs.fr

KEYWORDS:
Circulating tumor cells (CTC), liquid biopsy, 3D culture, drug screening, preclinical mouse model.

SUMMARY:
Culturing CTCs allows a deeper functional characterization of cancer, through assaying specific marker expression, and assessing drug resistance and the ability to colonize the liver among other possibilities. Overall, CTC culture could be a promising clinical tool for personalized medicine to improve patient outcome.

ABSTRACT:
Metastasis is a leading cause of cancer death. Despite improvements in treatment strategies, metastatic cancer has a poor prognosis. We thus face an urgent need to understand the mechanisms behind metastasis development, and thus to propose efficient treatments for advanced cancer. Metastatic cancers are hard to treat, as biopsies are invasive and inaccessible. Recently, there has been considerable interest in liquid biopsies including both cell-free circulating deoxyribonucleic acid (DNA) and circulating tumor cells from peripheral blood and we have established several circulating tumor cell lines from metastatic colorectal cancer patients to participate in their characterization. Indeed, to functionally characterize these rare and poorly described cells, the crucial step is to expand them in suspension. Once established, circulating tumor cell (CTC) lines can then be cultured in suspension or adherent conditions. At the molecular level, CTC lines can be further used to assess the expression of specific markers of interest (such as differentiation, epithelial or cancer stem cells) by immunofluorescence or cytometry analysis. In addition, CTC lines can be used to assess drug sensitivity to gold-standard chemotherapies as well as to targeted therapies. The ability of CTC lines to initiate tumors can also be tested by subcutaneous injection of CTCs in immunodeficient mice.

Finally, it is possible to test the role of specific genes of interest that might be involved in cancer dissemination by editing CTC genes, by short hairpin ribonucleic acid (shRNA) or Crispr/Cas9. Modified CTCs can thus be injected into immunodeficient mouse spleens, to experimentally mimic part of the metastatic development process *in vivo*.

In conclusion, CTC lines are a precious tool for future research and for personalized medicine, where they will allow prediction of treatment efficiency using the very cells that are originally responsible for metastasis.

INTRODUCTION:
Despite recent improvements in early cancer diagnosis and in therapeutic strategy, more than ninety percent of cancer morbidity is still due to metastasis[1]. The metastatic process is a multi-step cascade that starts with the local detachment of cells from the primary tumor and their

entrance into the bloodstream where they become circulating tumor cells (CTCs) to finally colonize distant sites such as liver and lungs, in the case of colorectal cancer (CRC) [2]. Recently, there has been growing attention to liquid biopsies, which are a non-invasive tool to notably detect and enumerate CTCs from patient blood samples. Intratumor genetic heterogeneity is a major cause of drug resistance; thus, isolating representative cells from tumor material constitutes a promising tool for personalized medicine[3].

Despite low frequency of CTCs in the blood (1 CTC per $10^6 – 10^7$ leukocytes)[4], several detection and isolation techniques were developed based on property differences between CTCs and other components of the blood[5]. The number of CTCs in patient blood samples, alone, can provide information about the stage of malignancy, treatment response and disease progression[6,7]. Thus, CTC isolation is a crucial tool for translational studies to assess genetic heterogeneity or perform drug screening, as well as for fundamental studies to characterize these invasive cells, as they are the key actors of metastatic induction[8,9]. Indeed, compared to commercially established cancer cell lines that have accumulated thousands of mutations over time, fresh CTCs share the main features of the original primary tumor including a potent capacity to metastasize, and they are a better reflection of the disease. These features make them a robust tool for fundamental studies, especially in knockout experiments of predicted key factors involved in metastasis. The outcome of these experiments can be validated *in vivo*, on mice, as described below.

Once CTCs are isolated, they can be expanded in non-adherent culture conditions and then, they can be manipulated just as any available cancer cell line, i.e. they can equally be cultured in adherent conditions or embedded in Matrigel, depending on the scientific question [10]. For example, to test the expression and localization of a protein of interest, CTC spheres can be grown in suspension condition and be embedded in Histogel to perform immunofluorescence on sphere sections. In addition, if the protein is membranous, its expression on living cells can be measured by cytometry.

For functional studies, to test the role of a protein of interest that may play a role in liver colonization, CTCs with genes edited, by shRNA or CRISPR/Cas9, can be injected into the spleen of immunodeficient mice. This latter experiment is a powerful model to mimic liver metastasis colonization [11].

The ability of CTCs to initiate tumors can be assessed by injecting a very small number of cells into immuno-deficient mice. As tumor initiation is a hallmark of cancer stem cells (CSCs) this assay will indicate the percentage of CSCs within CTC lines. This stem cell phenotype makes circulating tumor cell lines resistant to some gold-standard cancer therapies. Expanded CTCs can therefore be used to screen drugs and pinpoint the best potential efficient treatment for the patient; CTC response to treatment can be tested in vitro using a luminescence viability assay, for example.

In a long-term perspective, drug screening on freshly isolated and amplified CTCs could be used as a new tool for personalized medicine to aid in choosing the most efficient and adapted treatment for patients.

In the present paper, protocols to culture CTC lines, to stain specific proteins via immunostaining and cytometry, to perform cytotoxicity assays as well as in vivo xenograft experiments with CTC are detailed.

PROTOCOL:

All in vivo protocols were approved by the animal ethical agencies.

1. CTC Amplification in 3D Culture Conditions

1.1. To culture CTCs in suspension, first seed CTCs in wells of an Ultra-Low Attachment (ULA) 24-well plate at the maximal concentration of 5 cells/µL and into 1 mL of M12 medium (i.e., advanced DMEM-F12 supplemented with 2 mM L-glutamine, 100 Unit/mL penicillin and streptomycin, N2 supplement, 20 ng/mL epidermal growth factor and 10 ng/mL fibroblast growth factor [10]).

1.2. To allow sphere formation with CTC lines, incubate the cells in hypoxic conditions (2% O_2) between 6 to 10 days.

1.3. Once the spheres are large enough (100 µm), to prevent necrosis, dissociate them for amplification.

1.3.1. Place the cell suspension in a 2 mL tube or a 15 mL tube and centrifuge at 300 x g for 5 minutes.

1.3.2. Remove the supernatant and add 500 µL of gentle dissociation reagent (e.g., Accumax) to the pellet.

1.3.3. Vortex gently and incubate the cell suspension with gentle dissociation reagent for 20 to 30 minutes at 37 °C.

NOTE: Exceeding the incubation time in the gentle dissociation reagent (Accumax) may cause abnormal increased cell mortality.

1.3.4. Add 1.5 mL of Phosphate Buffered Saline (PBS) and centrifuge the tube for 5 minutes at 300 x g.

1.3.5. Pour off the supernatant and resuspend the pellet in fresh M12 medium to reach the density of 1-5 cells per µL.

1.3.6. Seed the cell suspension in new wells of an Ultra-Low Attachment plate: 10 mL for T75 flasks, 2 mL for 6 well plates and 100 µL for 96 well plates

2. Immuno-Fluorescence (IF) Staining on CTC Sphere Sections

2.1. Centrifuge CTC spheres into a 15 mL tube at 300 x g for 5 min, aspirate the supernatant, and wash the pellet twice with PBS.

2.2. Resuspend the pellet with 500 µL of 4% paraformaldehyde (PFA) and incubate for 20 min on ice. Centrifuge the suspension at 300 x g and wash twice with 5 mL of PBS.

2.3. Add a droplet of hot and liquid Histogel (i.e., 15 to 20 µL) on the pellet and mix well. With a pipette, take all the suspension and make a droplet on a precooled surface at 4 °C and let it polymerize for 5 to 10 minutes.

NOTE: Work fast to avoid the polymerization of the suspension into the tip.

2.4. Detach the solid droplet with the blade of a scalpel and insert it into a compartmented embedding cassette.

2.5. Perform the following steps: paraffin inclusion, paraffin sectioning, section rehydration, antigen retrieval and antibody incubation. These steps are the same as for any tumor or organ embedded in paraffin and processed for immunofluorescence staining[12].

NOTE: The cassette can be stored in 70% ethanol at 4 °C until paraffin embedding.

3. Cytometry Analysis

3.1. Centrifuge CTC spheres into a 15 mL tube at 300 x g for 5 min and wash the pellet twice with PBS.

3.2. Aspirate the supernatant and add 500 µL of the gentle dissociation reagent to the pellet.

3.3. Vortex gently and incubate the cell suspension with the gentle dissociation reagent for 20 to 30 min at 37 °C.

NOTE: Exceeding 40 min of incubation in the gentle dissociation reagent (Accumax) may result in an abnormal increased cell mortality.

3.4. Centrifuge the cell suspension for 5 min at 300 x g, eliminate the supernatant and resuspend the pellet in 5 mL of blocking buffer (i.e., PBS with 1% bovine serum albumin (BSA)).

3.5. Pass the cell suspension through a 40 µm cell strainer to eliminate the remaining spheres.

3.6. After counting, put 200,000 cells into three fluorescence-activated cell sorting (FACS) tubes, centrifuge at 300 x g for 5 min and remove the supernatant. Use one of the three FACS tube as a control that will not receive any staining reagents.

3.7. According to the datasheet, add the appropriate antibody into the two remaining FACS tubes (i.e., one conjugated antibody against the protein of interest, one conjugated isotype control).

3.8. After the recommended incubation time, add 300 µL of the blocking buffer to wash the cells, centrifuge the 3 tubes at 300 x g and aspirate the supernatant. Repeat this step twice.

Then, resuspend the pellet with the blocking buffer with a cell viability staining reagent (except for the FACS tube control without any staining).

3.9. Keep the tubes on ice until FACS analysis. Use first the tube without staining to identify the gate for the cell viability and the antibody staining. Then analyze the FACS tube with the conjugated antibody against the protein of interest, to quantify the percentage of CTC expressing the protein of interest. The FACS tube with the conjugated isotype control should not indicate a shift. This confirms that the shift is specific to the targeted protein of interest.

NOTE: If possible, use a cell line expressing a high level of protein of interest as a positive control and a cell line that does not express the protein as a negative control.

4. Luminescence Viability Assay (CellTiter-Glo)

4.1. Resuspend dissociated CTCs (as described in section 1.4.5) in M12 medium. Count the cells and adapt the cell concentration to 200,000 cells/mL.

4.2. In a ULA 96-well plate, seed 10,000 dissociated CTCs per well, in 50 µL, and incubate the plate in hypoxia (2% O_2) for 24 h.

4.3. The following day, add 50 µL of the tested drug to the CTCs. It is previously recommended to perform a titration of the drug concentration, to determine the half maximal inhibitory concentration (IC50). Treat some wells with vehicle only to determine basal cell viability.

NOTE: The number of cells seeded may differ between CTC lines, depending on their doubling time. In addition, cells should be seeded in triplicates to minimize results variability.

4.4. Incubate plates in hypoxia for another 48 h.

4.5. At day 3, place the cultured plates and the luminescence cell viability buffer and substrate at room temperature and reconstitute the substrate with an appropriate volume of buffer, according to manufacturer protocol.

4.6. Add 70 µL of the luminescence viability mix into each cultured plate well. Put plates on an orbital shaker for 20 min to induce cell lysis and then transfer 100 µL of the mix into an opaque-walled multi-well plate, compatible with the luminometer.

4.7. Allow the plate to rest at room temperature, cover it with aluminum foil in order to stabilize the luminescent signal.

NOTE: Luminescent signal is stable for up to 3 hours.

4.8. Record luminescence using a microplate reader (i.e., Tecan Infinite 200 Pro).

4.9. For data analysis, calculate the mean of the triplicate relative light unit RLU per condition. To assess the percentage of viability according to each drug concentration needed: (RLU of treated cells/RLU of untreated cells) x 100.

5. Subcutaneous Injections

5.1. In a microcentrifuge tube, resuspend cells in 100 µL of sterile PBS. If the number of injected cells is low, resuspend cells in 1:1 PBS/Matrigel, reduced in growth factors, to concentrate cells together and promote tumor initiation.

NOTE: Cell density can be from 10 cells (to challenge the tumor initiation capacity) to 1 million of cells depending on the scientific question and the aim of the experiment.

5.2. Pull up the full volume in an insulin syringe.

5.3. On an immune-deficient mouse, pinch the skin of the flank between the index and the ring finger and gently insert the needle at the base of the skin.

NOTE: This procedure is not painful and is done on conscious mice.

5.4. Slowly inject the volume in the same spot to prevent the spreading of the cells. This will create a small bleb beneath the skin.

5.5. Apply gentle pressure on the injection site to prevent the backflow of the volume.

5.6. Monitor the tumor growth, every other day, using a caliper.

5.7. Sacrifice the animal if the tumor exceeds 1500 mm^3. Depending on the material availability, euthanize the mice by carbon dioxide or anaesthetic gases inhalation or by cervical dislocation.

6. Intrasplenic Injection

6.1. Before starting, autoclave the surgical instruments (scissors and forceps) at 124 °C for 15 min and clean out all surfaces of the working space with 70% ethanol.

6.2. In a microcentrifuge tube, resuspend cells in 50 µL of sterile PBS and store them on ice. Cell density can go from 0.5 to 1 million cells. A larger number of cells can cause a clots development into the blood circulation.

6.3. Prior to injecting the cells, inject 100 µL of 0.015 mg/mL buprenorphine subcutaneously, for pain relief.

6.4. Place the mouse in an induction chamber, where isoflurane is maintained at 3%, for 2-3 min. When the animal is no longer responsive to movement, put it on its right-hand side on a heating pad and maintain the anaesthesia using a breathing circuit face mask that delivers a gas mixture of O_2 and isoflurane.

6.5. Apply eye lubricant over each eye to avoid eye drying during the surgery. Disinfect the thoracic area with 70% ethanol.

6.6. On the dorsoventral side, make a small incision of 0.5 cm using scissors below the 10th false rib.

6.7. Gently lift the spleen out and lay it on sterile gauze. Using an insulin syringe, inject the 50 µL of CTCs in the tip of the spleen. Make sure to maintain the syringe upright and wait 3-5 min to avoid backflow, and then remove the needle.

6.8. Ligate the splenic vessels from the two sides (arteries and veins) and remove the spleen by cutting directly above the two ligatures. The spleen is removed to prevent unwanted tumor formation in this organ.

6.9. Stitch the abdominal peritoneum and then the skin using 5.0 degradable sterile sutures.

6.10. Keep the mouse warm until its full recovery from anesthesia.

6.11. Monitor the mouse 3 days per week for abnormal bodily function, abnormal movement and posture and for weight loss.

6.12. Sacrifice the animal when humane end points reach (approximately 6 weeks after the surgery) to analyze liver metastasis.

REPRESENTATIVE RESULTS:

Both EpCAM and CD26 expressions observed by IF (**Figure 1A**) and FACS (**Figure 2A**) respectively, indicate that the CTC line is epithelial and display one of the CSC hallmarks[10]. This epithelial trait can be further characterized by staining with antibodies directed against other epithelial and mesenchymal markers. Thereby, it could be possible to approximately know where the CTC line is along the epithelial-mesenchymal axis. Expression of other CSC markers could be also tested by both immunostaining and cytometry. Otherwise, functional tests as drug resistance and tumor initiating assay in vivo can validate this CSC phenotype[10]. Here for example, luminescence viability assay shows that the CTC IC50 is only reached at high drug concentration highlighting the high resistance of these cells (**Figure 2**). Moreover, subcutaneous CTC injection shows that this CTC line has tumorigenic ability (**Figure 3A**). These latter results confirm the CSC phenotype of this CTC line. Challenging the tumor initiation capacity by injecting from 10 to 10,000 cells can refine the estimation of the tumorigenic ability. Finally, liver metastasis formation by mimicking dissemination through intrasplenic injection shows that this CTC line can survive in a distant organ and has a metastatic potential (**Figure 3B**). This metastatic potential can be compared to other cell lines or challenged by inhibiting specific gene expression or upon chemotherapy administration in mice after intrasplenic injection.

Figure 1: Microscopy and Flow cytometry analysis of CTC spheres show that they expressed both epithelial and CSC markers. (**A**) Left panel: CTC spheres cultured for 6 days before embedding. Right panel: epifluorescence microscopy analysis of embedded CTC spheres in Histogel. 5 µm sections of embedded CTC spheres were stained by an antibody against the epithelial marker Epithelial cell adhesion molecule (EPCAM) (in red) and the nucleus is labelled with DAPI (in blue). Scale bar is 20 µm. (**B**) Flow cytometry analysis of alive single CTCs. Upper

left FACS plot shows CTCs without labelling to gate cells based on their size and granularity. Upper right FACS plot shows CTCs with a viability marker to gate alive cells only. Bottom left FACS plot shows CTCs labelled with APC-conjugated isotype control to gate positively labelled cells. Bottom right FACS plot shows CTCs labelled by APC-conjugated CD26 antibody to assess the percentage of cells expressing this CSC marker (67%).

Figure 2: Representative result of the cell growth assay using a luminescence viability assay. 10,000 CTCs were seeded per well and were then treated with an increasing concentration of the molecule of interest for 72 hours. The IC50 is reached at x µM.

Figure 3: CTCs have a tumorigenic and metastatic potential. (**A**) Subcutaneous injection of 200,000 CTCs on the right flank of Nude mice. Photos were taken 1 month after the injection and tumor size was measured 3 times per week. (**B**) Intrasplenic injection of CTCs to mimic liver metastatic colonization. Right panel: representative photo of mouse liver dissected 4 weeks after intrasplenic injection of 300,000 CTCs. Left panel: mouse liver dissected 4 weeks after intrasplenic injection of PBS.

DISCUSSION:

The protocol described above was used initially for colorectal CTC functional characterization, but it can be used for other types of cancer such as breast cancer and can be adapted for mouse models.

The real limiting factor is the number of CTCs present in the blood sample and the efficiency of the technique used to isolate and expand them. Several CTC isolation technics have been described based on specific CTC properties such as the Parsortix, a microfluidic device, that allows the isolation of CTCs based on the cell size and its compressibility[13]. In addition, there is the CellSearch, an immunobead based assay that is the only FDA-approved method to enumerate CTCs in blood samples based on a negative CD45 labeling to eliminate contaminating lymphocytes combined with positive epithelial marker labelling such as EpCAM and cytokeratin 8/18/19[14]. The iChip is another size-based technology but it also combines the CTC enrichment using either an EpCAM based positive selection or CD45 negative depletion[15,16]. Finally, we have recently used the fast and easy immunodensity procedure, Rosette sep kit, to isolate and amplify CTC from the colorectal cancer patient blood samples[10].

If working on a mouse model, it is also possible to pool mouse blood samples from the same cohort to increase the number of CTCs and the chance of amplifying them in culture. In the case of murine CTCs, it is not mandatory to use immunodeficient mice to test the liver colonization by intrasplenic injection. Control mice with the same genetic background can be used as recipient mice without any impact of the immune response.

Once CTCs are amplified in suspension, they can be compared with other cancer cell lines from the same type of cancer using each technique described here. In this case, different cancer cell lines must be cultivated in the same conditions. Particular attention must be paid to innate auto-fluorescence, for the immunofluorescence staining and cytometry analysis. The CTCs and each cancer cell line used must be tested without any staining in the different fluorescent channels.

If the CTCs under study can adhere and proliferate in adherent conditions like any classical cell line, then the techniques described above can be adapted for cells expanded in these conditions. The resulting readouts will likely thus be more relevant where the cells are from more differentiated cancer cell populations as cells tend to be less differentiated in suspension.

In conclusion, CTC lines are very precious tools to deeply characterize mechanisms involved in metastatic processes leading cancer lethality, and in the future, as culture success rates improve CTCs could be used to propose the best therapeutic strategy adapted to each patient.

ACKNOWLEDGMENTS:

This research project in the Pannequin lab was supported by research grant from the SIRIC: Grant « INCa-DGOS-Inserm 6045 ». The PhD theses of Guillaume Belthier and Zeinab Homayed were supported by the anti-cancer league/Ligue contre le Cancer. Céline Bouclier salary was financed by “region Occitanie”. Thanks to Julian Venables for English editing.

DISCLOSURES:

The authors have no competing interests to disclose.

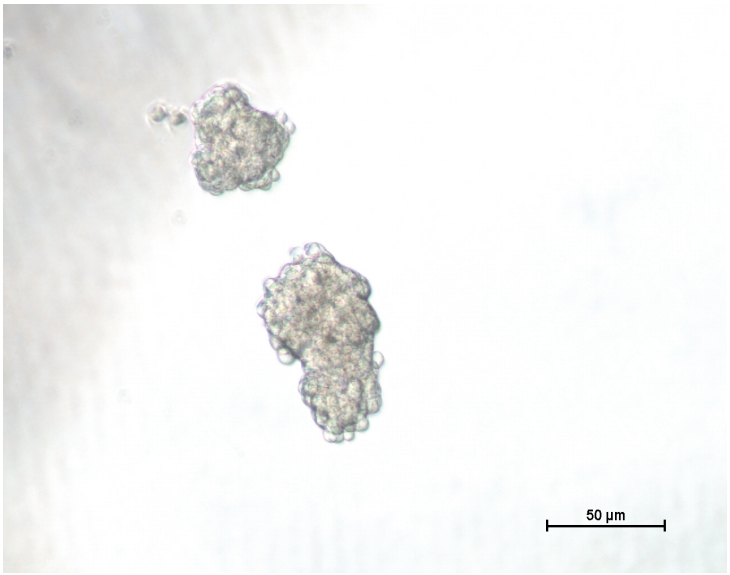
REFERENCES:

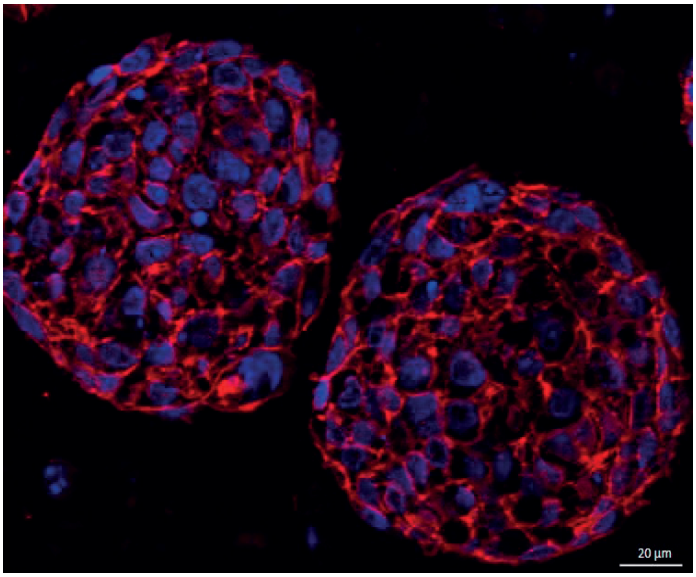
1. Wittekind, C., Neid, M. Cancer invasion and metastasis. *Oncology*. **69 Suppl 1**, 14–16 (2005).
2. Eger, A., Mikulits, W. Models of epithelial–mesenchymal transition. *Drug Discovery Today: Disease Models*. **2**, 57–63 (2005).
3. Palmirotta, R. *et al.* Liquid biopsy of cancer: a multimodal diagnostic tool in clinical oncology. *Therapeutic Advances in Medical Oncology*. **10**, (2018).
4. Hong, B., Zu, Y. Detecting circulating tumor cells: current challenges and new trends. *Theranostics*. **3**, 377–394 (2013).
5. van der Toom, E. E., Verdone, J. E., Gorin, M. A., Pienta, K. J. Technical challenges in the isolation and analysis of circulating tumor cells. *Oncotarget*. **7**, 62754–62766 (2016).
6. Jin, L. *et al.* Evaluation of the diagnostic value of circulating tumor cells with CytoSorter® CTC capture system in patients with breast cancer. *Cancer Medicine*. **9**, 1638–1647 (2020).
7. Huang, X. *et al.* Relationship between circulating tumor cells and tumor response in colorectal cancer patients treated with chemotherapy: a meta-analysis. *BMC Cancer*. **14**, 976 (2014).
8. Yu, M. *et al.* Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. *Science*. **345**, 216–220 (2014).
9. Aceto, N. *et al.* Circulating Tumor Cell Clusters are Oligoclonal Precursors of Breast Cancer Metastasis. *Cell*. **158**, 1110–1122 (2014).
10. Grillet, F. *et al.* Circulating tumour cells from patients with colorectal cancer have cancer stem cell hallmarks in ex vivo culture. *Gut*. **66**, 1802–1810 (2017).
11. Lee, W. Y., Hong, H. K., Ham, S. K., Kim, C. I., Cho, Y. B. Comparison of Colorectal Cancer in Differentially Established Liver Metastasis Models. *Anticancer Research*. **34**, 3321–3328 (2014).
12. Giraud, J. *et al.* Progastrin production transitions from Bmi1+/Prox1+ to Lgr5high cells during early intestinal tumorigenesis. *Translational Oncology*. **14**, (2020).

13. Xu, L. *et al.* Optimization and Evaluation of a Novel Size Based Circulating Tumor Cell Isolation System. *PLoS One*. **10**, e0138032 (2015).
14. Beije, N., Jager, A., Sleijfer, S. Circulating tumor cell enumeration by the CellSearch system: the clinician's guide to breast cancer treatment? *Cancer Treatment Reviews*. **41**, 144–150 (2015).
15. Karabacak, N. M. *et al.* Microfluidic, marker-free isolation of circulating tumor cells from blood samples. *Nature Protocols*. **9**, 694–710 (2014).
16. Ozkumur, E. *et al.* Inertial Focusing for Tumor Antigen–Dependent and –Independent Sorting of Rare Circulating Tumor Cells. *Science Translational Medicine*. **5**, 179ra47 (2013).

Figure 1

A





B

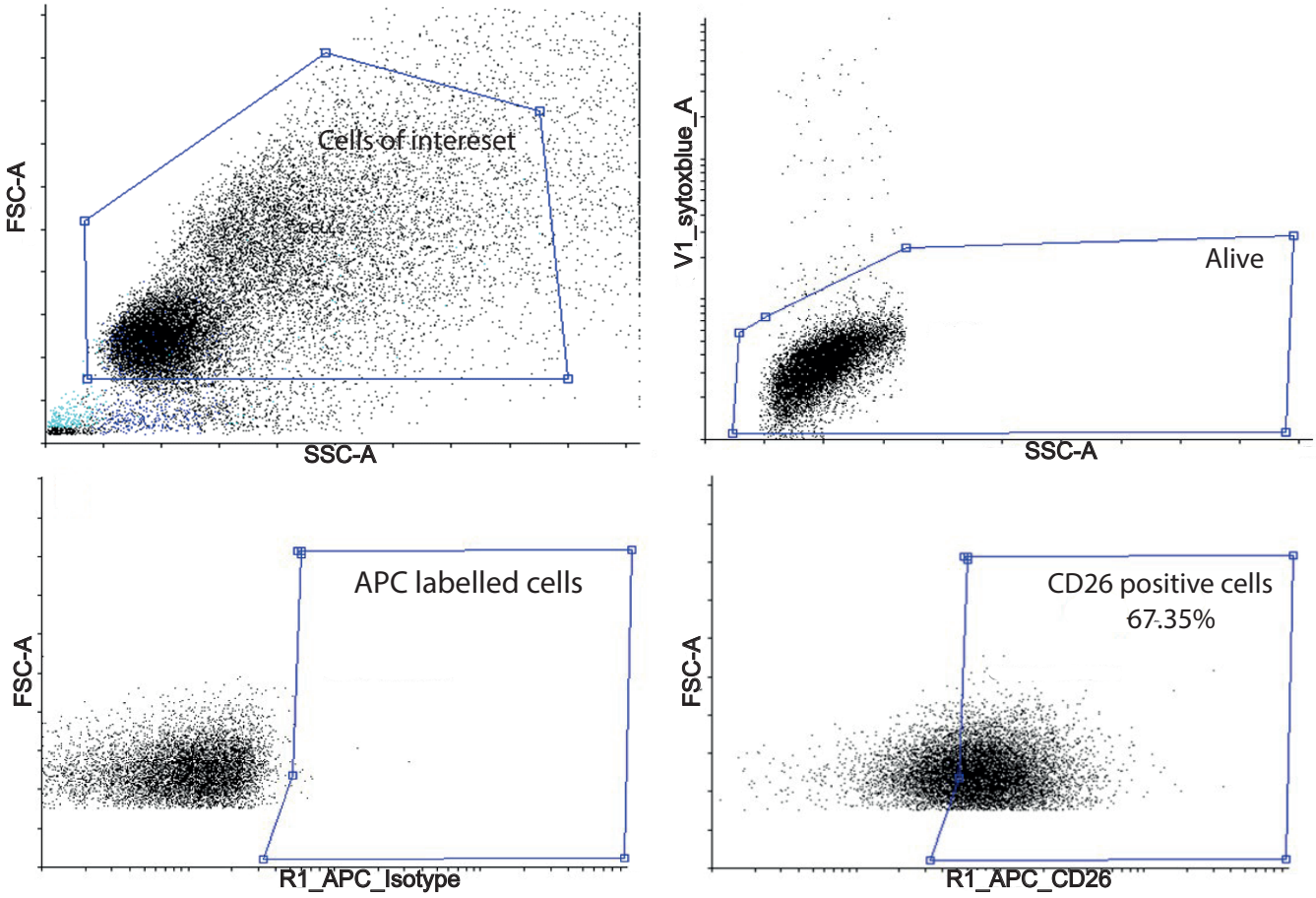


Figure 2

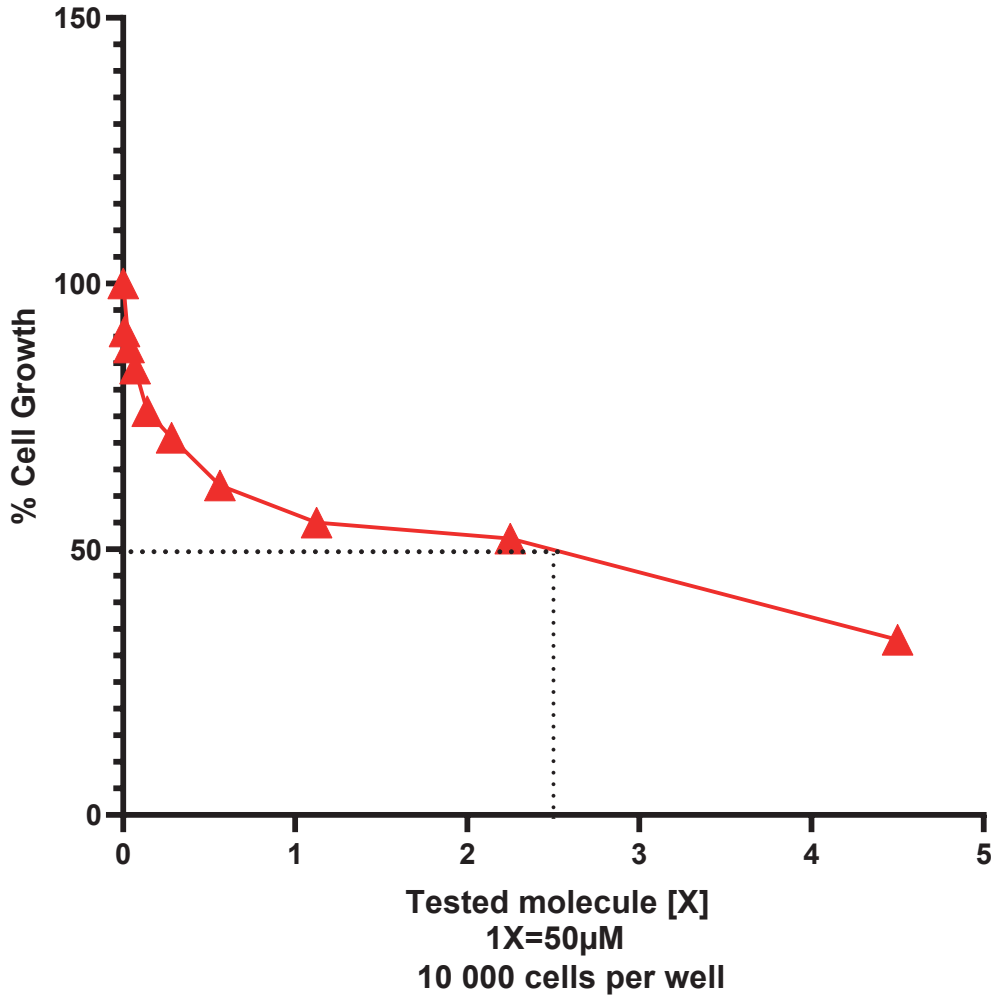
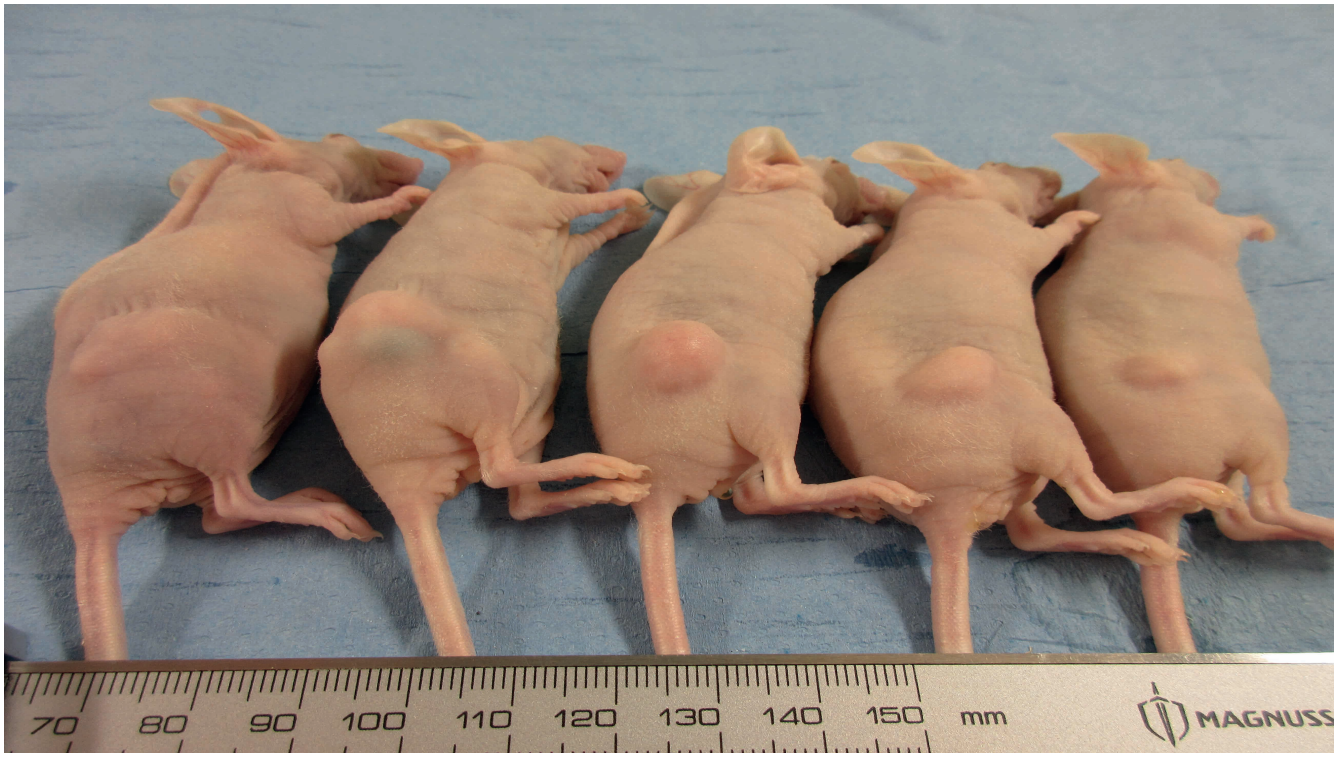


Figure 3

A



B

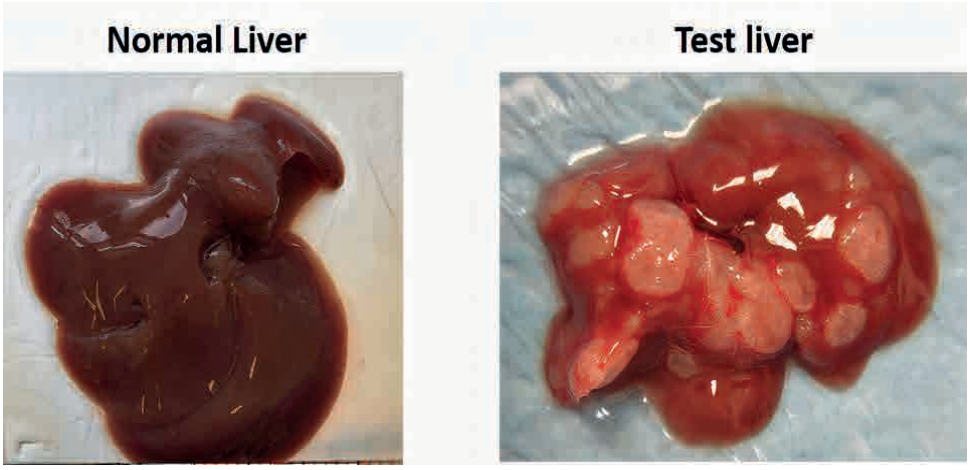


Table of Materials

INSTITUT DE GÉNOMIQUE FONCTIONNELLE

CNRS UMR5203 - INSERM U1191 - UNIVERSITÉ DE MONTPELLIER

Pannequin Julie
Team leader of the « Signaling, Plasticity and Cancer » team
CNRS UMR5203, INSERM U1191 et UM
141, rue de Cardonille
F-34094 Montpellier
04 34 35 92 65
julie.pannequin@igf.cnrs.fr

Object : Rebuttal letter (3)

Montpellier the 6th of august 2021,

Dear editors
We thank you for the opportunity to submit a revised version of our manuscript JoVE62329R3 entitled " Circulating tumor cell lines: an innovative tool for fundamental and translational research ", by G. Belthier and colleagues.

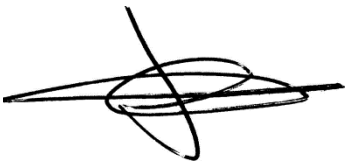
We thank the editorial board for helping us to improve the video.

All editorial comments have been addressed in the revised version, as detailed below in the point-by-point response to the editorial' comments that you will find in blue font.

We trust that the originality, the quality and the significance of this work make it a worthwhile candidate for publication in your Journal and we thank you in advance for considering this revised version.

Best Regards,

Julie Pannequin



Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:
None.
Authors would like to aknowledge and editors for this absence of comments regarding the written manuscript
Changes to be made by the Author(s) regarding the video:
1. Title Cards:
• Please capitalize the first letter of word ""lines"" in your main title card. i.e, ""Lines""."

This change has been made
• 1:14 - Please do not capitalize by (by instead of By) and add a period here.
This modification has been done
2. Video Editing Content:
• 04:24 Please remove the Dip to black tranisition.

As suggested authors have removed this dip to black transition
• Please combine all the results sections into one single section and place it after Protocols and before Conclusion section. This is required to fit our publication standard as the results are presented together in the written manuscript as well. For more details, please see the attached guide.

Please find below discussion between Nam Nguyen and myself regarding the latter comment, authors have thus decided not to address this point:

Myself:

"Dear Nam
I thank you for your rapid feedback. We will address the minor comments tomorrow and submit the revised version in the mean time. However concerning this particular point " Please combine all the results sections into one single section and place it after Protocols and before Conclusion section. This is required to fit our publication standard as the results are presented together in the written manuscript as well." it is strictly impossible for us to change this. First as I said in the latest rebuttal letter it would necessitate a huge amount of time and a complete new record of the voice which is impossible since people is on holidays, in September many experiments are already planned on mice and cannot be delayed, Guillaume Belthier has left the lab pour a post doctoral fellowship and we do not have access anymore to the equipment used for recording. Second it is not logical to pool all results together since we have 5 different types of experiments, how do you want us to be didactic by showing tumors and microscopy images in a raw. You gave the example of the manuscript but it is different cause you can turn the page or scroll on internet to have a view on the cited image, here you need to stop the movie and the interruption will prevent fluidity in the story. Finally we found examples of videos published previously by JoVE in which this organization has been followed (https://www.jove.com/v/62373/generation-of-zebrafish-larval-xenografts-and-tumor-behavior-analysis*

Institut de Génomique Fonctionnelle
141 rue de la Cardonille - Montpellier - F-34094 Cedex 5 – France
Tel : +33 (0) 4 34 35 92 65 Fax : +33 (0) 4 67 54 24 32
Courriel : julie.pannequin@igf.cnrs.fr

https://www.jove.com/v/62065/creation-and-maintenance-of-a-living-biobank-how-we-do-it).
I would be happy to discuss with you if you need but believe me this change is not feasible and will worsen the quality of our video.
Sincerely yours

Nam's response

Hi Julie,

Thanks for reaching out.

Please address the rest of the comments regarding the video revisions.

Regarding the collation of the results, I will talk to our video team
but I think we may be able to work something out.

Please submit the video with the other comments addressed.

Nam

Institut de Génomique Fonctionnelle
141 rue de la Cardonille - Montpellier - F-34094 Cedex 5 – France
Tel : +33 (0) 4 34 35 92 65 Fax : +33 (0) 4 67 54 24 32
Courriel : julie.pannequin@igf.cnrs.fr