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Chick heart invasion assay for testing the invasiveness of cancer cells and the activity of potentially anti-invasive compounds.

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Abstract:	The goal of the chick heart assay is to offer a relevant organ culture method to study tumor invasion in three dimensions. The assay can distinguish between invasive and non-invasive cells, and allows to study the effects of test compounds on tumor invasion. Cancer cells - either as aggregates or single cells - are confronted with fragments of embryonic chick heart. After organ culture in suspension for a few days or weeks the confronting cultures are fixed and embedded in paraffin for histological analysis. The three-dimensional interaction between the cancer cells and the normal tissue is then reconstructed from serial sections stained with hematoxylin-eosin or after immunohistochemical staining for epitopes in the heart tissue or the confronting cancer cells. The assay is consistent with the recent concept that cancer invasion is the result of molecular interactions between the cancer cells and their neighbouring stromal host elements (myofibroblasts, endothelial cells, extracellular matrix components…). In the assay this stromal environment is offered to the cancer cells as a living tissue fragment. Supporting aspects to the relevance of the assay are multiple. Invasion in the assay is in accordance with the criteria of cancer invasion: progressive occupation and replacement in time and space of the host tissue, and invasiveness or non-invasiveness in vivo of the confronting cells generally correlates with the outcome of the assay. Furthermore, the invasion pattern of cells in vivo, as defined by pathologists,

	is reflected in the histological images in the assay. Quantitative structure-activity relation (QSAR) analysis of the results obtained with numerous potentially anti-invasive organic congener compounds allowed the study of structure-activity relations for flavonoids and chalcones, and known anti-metastatic drugs used in the clinic (e.g. microtubule inhibitors) inhibit invasion in the assay as well. However, the assay does not take into account immunological contributions to cancer invasion.
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Dear Editor,

We would like to submit the manuscript entitled “Chick heart invasion assay for testing the invasiveness of cancer cells and the activity of potentially anti-invasive compounds” for publication in the Journal of Visualized Experiments.

The assay is based on confronting organ cultures of embryonic chick heart fragments and aggregates of cancer cells. This implies a number of critical manipulations that are more easily explained by video demonstration than by static description. The assay was introduced by us in the late seventies, and has been applied since then in several other labs. Although short descriptions of the assay steps were published, successful implementation in those labs generally required a short visit and demonstration in our lab. We feel that publication in JoVE would greatly facilitate the dispostion of the assay to potentially interested users.

The contributions of the co-authors were as follows:
Marc Bracke: laboratory director, supervisor of the assay and author of the manuscript
Bart Roman, Christian Stevens, Liselot Mus and Virinder Parmar: long-term scientific users of the assay, who have improved the procedure and established structure-activity relations of anti-invasive compounds. They defined the robustness of the assay over the last 35 years (see manuscript).
Olivier De Wever: supervisor of different invasion assays in our laboratory, responsible for the performance comparisons between the assays.
Marc Mareel: author of the original publications of the chick heart invasion assay in our lab, and critical reader/revisor of the manuscript.

Nandita Singh was the JoVE editor who assisted me in the submission process.

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Thank you for considering the publication of our manuscript in JoVE.

Sincerely yours

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KEYWORDS:
cancer, invasion, organ culture, histology, heart, chicken, ecosystem, anti-invasive compound, polyphenolics, structure-activity, immunohistochemistry.

SHORT ABSTRACT:
Here, we present a protocol to study the invasion of tumor cells into living normal tissue fragments in three dimensions. This organ culture technique is mainly applied to test potentially anti-invasive drugs *in vitro*.

LONG ABSTRACT:
The goal of the chick heart assay is to offer a relevant organ culture method to study tumor invasion in three dimensions. The assay can distinguish between invasive and non-invasive cells, and enables study of the effects of test compounds on tumor invasion. Cancer cells - either as aggregates or single cells - are confronted with fragments of embryonic chick heart. After organ culture in suspension for a few days or weeks the confronting cultures are fixed and embedded in paraffin for histological analysis. The three-dimensional interaction between the cancer cells and the normal tissue is then reconstructed from serial sections stained with hematoxylin-eosin or after immunohistochemical staining for epitopes in the heart tissue or the confronting cancer cells. The assay is consistent with the recent concept that cancer invasion is the result of molecular interactions between the cancer cells and their neighbouring stromal host elements (myofibroblasts, endothelial cells, extracellular matrix components, etc.). Here, this stromal environment is offered to the cancer cells as a living tissue fragment. Supporting aspects to the relevance of the assay are multiple. Invasion in the assay is in accordance with the criteria of cancer invasion: progressive occupation and replacement in time and space of the host tissue, and invasiveness and non-invasiveness *in vivo* of the confronting cells generally correlates with the outcome of the assay. Furthermore, the invasion pattern of cells *in vivo,* as defined by pathologists, is reflected in the histological images in the assay. Quantitative structure-activity relation (QSAR) analysis of the results obtained with numerous potentially anti-invasive organic congener compounds allowed the study of structure-activity relations for flavonoids and chalcones, and known anti-metastatic drugs used in the clinic (*e.g.* microtubule inhibitors) inhibit invasion in the assay as well. However, the assay does not take into account immunological contributions to cancer invasion.

INTRODUCTION:
Invasion is the hallmark of malignant tumors. This activity not only leads to the destruction of surrounding tissues, but is also implicated in metastasis formation. Since cancer patients die from invasion and metastasis, and efficient anti-invasive treatments are still scarce, laboratory assays that mimic the invasion of tumor cells have been developed. The goal of the chick heart assay is to offer a relevant organ culture method to study tumor invasion in three dimensions. The assay can distinguish between invasive and non-invasive cells, and allows to study the effects of test compounds on tumor invasion.

The rationale behind the use of the assay is the actual concept that tumors are ecosystems where the neoplastic cells continuously interact with their stroma (host cells and extracellular matrix), and that through these molecular interactions invasion is fine-tuned[1]. So, in the assay tumor cells are confronted with living embryonic chick heart fragments[2], which serve not only as substrates for invasion by the tumor cells, but also as a source of different types of stromal cells and matrix elements. The chick heart contains myocytes, fibroblasts and

endothelial cells, and the extracellular matrix is composed of laminin, fibronectin and different types of collagen. In this way, the three-dimensional organ culture technique covers many cellular and molecular interactions implicated in invasion of patient tumors.

The main advantage of the chick heart assay is the implementation of stromal effects. This aspect is more complete than in other invasion assays *in vitro* that are based on tumor cell invasion into non-living gels composed of basement membrane[3] or interstitial matrix[4] molecules. The concept of confrontation between tumor cells and normal living host tissue as found in organ culture experimentation has been introduced by several authors including Wolff and Schneider in France[5], Easty and Easty in the United Kingdom[6], and Schleich in Germany[7]. Two technical advantages of the chick heart invasion assay over the cited methods is that the volume of the fragments can easily be standardized, and that they remain contractile, which allows functional integrity monitoring during organ culture. Furthermore, avian embryos are preferred, because they can easily be dissected from the sterile content of the egg. The assay has conceptual resemblance to the chick chorioallantois membrane assay[8] by offering a complex stromal surrounding to the tumor cells.

The assay has successfully been applied to distinguish between invasive and non-invasive cell variants from the same human tumors such as in the MCF-7 (mammary)[9] and HCT-8 (colonic)[10] cell line families. The technique is useful to test potentially anti-invasive compounds as well[11,12]. As further explained, it can be used for the establishment of structure-activity relations of small organic molecules. The assay does, however, not take into account the contribution of immunological cells to cancer invasion. It should be stressed that the technique cannot be considered as a high-throughput analysis system, because of the high number of manipulations, the limited numbers of assay runs (maximum 30 cultures) and the long turn-around time (about 1 month).

PROTOCOL: (see figure 1)

[Place Figure 1 here]

1. Preparation of precultured heart fragments (PHFs)

1.1. Incubate a fertilized chick egg at 37 °C for 9 days. Complete this incubation by a date that allows subsequent preparation of PHFs during 4 days (*e.g.* on a Thursday) and final confrontation with tumor cell aggregates (*e.g.* on the nest Monday).

1.2. Disinfect the shell with 70% (v/v) ethanol in water. Carry out all further manipulations in a tissue culture cabinet using sterile solutions and materials. Open the shell at the embryonic pole using blunt forceps.

1.3. Pull out the embryo by holding the neck with an enucleation spoon (Figure 2). Place the embryo in a glass Petri dish with a diameter of 5 cm containing 5 mL of Ringer's salt solution.

1.3.1. Open the ventral thoracic skin by ripping open the skin using a sharp forceps in both hands, remove the sternum by the same type of manipulation, and dissect out the heart using microdissection iridectomy scissors to disconnect its major blood vessels. Perform all further manipulations under a macroscope with a calibrated ocular grid.

[Place Figure 2 here]

1.4. Transfer the heart to a glass Petri dish with a diameter of 5 cm containing 5 mL of MEM-Rega 3 culture medium with 5% fetal bovine serum. Remove the atria and associated vessels by resecting the upper cranial third of the heart using microdissection iridectomy scissors. Dissect the pericardium from the ventricles with a pair of sharp forceps.

1.5. Make a sagittal hemisection in the ventricles using microdissection iridectomy scissors, and remove the blood by gently shaking with a sharp forceps.

1.6. Transfer the ventricles into another glass Petri dish with a diameter of 5 cm containing 5 mL of fresh MEM-Rega 3 culture medium with 5% fetal bovine serum. Cut the ventricles into pieces of approximately 0.4 mm in diameter by using microdissection iridectomy scissors. One heart can yield about 100 fragments.

Note: One heart can yield about 100 fragments

1.7. Rotate the Petri dish gently to drive all ventricular fragments toward the center of the dish. Remove all corpora aliena with a stainless steel needle by separating them from the ventricular fragments and driving them towards the periphery of the Petri dish.

1.8. Transfer the heart fragments with a glass Pasteur pipet to a 50 mL Erlenmeyer flask containing 2 to 3 mL of culture medium (MEM-Rega 3 plus 10% v/v fetal bovine serum).

Note: The exact medium volume depends on the variable bottom convexities of the individual flasks: the centre of their bottom should be covered by a thin film of liquid only.

1.9. Gas the flask(s) with a mixture of 5% CO_2 in air via the stoppers, and incubate the flask(s) on a gyrotory shaker at 37 °C at 70 revolutions/min (rpm) for 24 hr. The rationale for this suspension culture step is to obtain spheroidal heart tissue fragments suitable for subsequent confrontation with tumor cell aggregates.

1.10. Transfer the heart fragments and their culture medium to a Petri dish. Discard corpora aliena, necrotic (dark) fragments, and conglomerates of heart fragments with a needle.

1.11. Incubate the remaining heart fragments in another 50 mL Erlenmeyer flask containing 6 mL of culture medium as described in step 1.9 for another 60 h.

Note: During this incubation period, the fragments will become spherical. They consist of a core of myoblasts and a thin layer of fibroblastic cells at the periphery.

1.12. Select spheroidal fragments exhibiting a thin, homogeneous layer of fibroblastic cells (forming a translucent capsule) and a diameter of 0.4 mm by means of a macroscope and needles. One chick heart will yield about 20 suitable PHFs. Transfer these PHFs, many of which will contract rhythmically at 37 °C, to another Petri dish containing fresh culture medium. These PHFs are ready for confrontation with test cells.

2. Preparation and confrontation of spheroidal test cell aggregates

2.1. Prepare semi-solid agar medium: dissolve 100 mg of agar in 15 mL of Ringer's salt solution by boiling three times. Cool the suspension to 40 °C and add 7.5 mL of Ringer's salt solution/egg white (1:1) and 7.5 mL of fetal bovine serum.

2.2. Prepare 6 mL of a suspension containing 1×10^5 test cells/ml in their appropriate culture medium in a 50 mL Erlenmeyer flask. Do this 3 days before the start of the confronting culture is planned. Incubate the flask on a gyrotory shaker at 37 °C at 70 rpm for 3 days. Gas the flasks with a mixture of 5% or 10% (v/v) CO_2 in air, depending on the type of culture medium used.

2.3. View the aggregates under a macroscope equipped with a calibrated ocular grid. Select (with a needle) spheroidal cell aggregates with a diameter of 0.2 mm.

2.4. Use a Pasteur pipet to transfer eight selected PHFs (diameter = 0.4 mm) in a minimal amount of culture medium to an embryological watch-glass containing semisolid agar medium[13]. Move the individual PHFs with a needle to form a circle. Aspirate excess medium using a small piece of filter paper (see Figure 3).

[Place Figure 3 here]

2.5. Bring ten selected spheroidal cell aggregates (diameter = 0.2 mm) into the circle using a Pasteur pipet. Aspirate excess medium. Move one aggregate towards each of the PHF with the needle until they make contact with each other. Aspirate excess medium using a small piece of filter paper.

2.6. Seal the lid of the watch-glass with paraffin, and incubate at 37 °C for 4 - 24 h, depending on the adhesive properties of the test cells to the PHFs.

2.7. Immerse the confronting pairs with pre-warmed (37 °C) culture medium, and transfer each individual pair with a Pasteur pipet into a 5 mL Erlenmeyer flask containing 1.5 mL of culture medium.

2.8. Incubate the flasks on a gyrotory shaker at 37 °C adjusted to 120 rpm. Gas the flasks with 5% or 10% (v/v) CO_2 in air, depending on the type of culture medium used for the test cells (see Figure 4).

2.8.1. To avoid concentration of the media, moisten the gasses by passing them through two supplementary 5 mL Erlenmeyer flasks filled with 2 mL of Ringer's salt solution. Refresh the culture medium every 8 days.

[Place Figure 4 here]

3. Histology of the confronting cultures

3.1. Prepare Bouin-Hollande's fixation solution.

3.1.1. Dissolve 2.5 g of cupric acetate (neutral) in 100 mL of single-distilled water and slowly add 4.0 g of picric acid.

3.1.2. Filter the solution through a paper filter. Add 10 mL of formalin and 1 mL of acetic acid. Mix 9 parts of this solution with 1 part of a saturated mercuric chloride solution in single-distilled water.

CAUTION: This solution contains several toxic substances. Formalin is toxic by inhalation, in contact with skin, and if swallowed. Acetic acid is corrosive for mouth and intestinal tract after ingestion. Picric acid is allergenic and explosive when rapidly heated or by percussion. Mercuric chloride is highly corrosive to mucous membranes and nefrotoxic. Wear protective clothing and gloves for preparing Bouin-Hollande's solution, and handle it in a well ventilated area remote from fire. An alternative fixation procedure is based on 4% formaldehyde in phosphate-buffered saline.

3.2. Fix the individual cultures after several days or weeks of incubation as follows. First, transfer the cultures to Ringer's salt solution for a few seconds to remove serum proteins. Next, immerse in Petri dishes with a diameter of 3 cm containing 3 mL Bouin-Hollande's fixation solution for approximately 2 h.

3.3. Rinse the cultures three times in 3 mL demineralized water before incubating them in water for 2 h to remove as much fixation solution as possible. Finally, transfer to 3 mL of 70% (v/v) ethanol in water. Keep the cultures in this solution overnight, but this can be extended for a number of days.

3.4. Dehydrate by transferring sequentially through jars containing 100 mL of 96% (v/v) ethanol in water, 100% ethanol or 100% isopropanol, and 100% xylene for 2 hr each.

3.4.1. Transfer each culture to a separate glass coverslip (with a minimal amount of xylene), place the coverslip on the bottom of a wine bottle capsule for paraffin embedding, and cover the fixed material with liquid paraffin wax at 56 °C. Incubate at 56 °C for 24 h, and then cool the embedded material to room temperature.

CAUTION: As a benzene derivative xylene may be toxic after inhalation and should be handled in well ventilated areas only.

3.5. Remove the wine bottle capsule and the coverslip, and cut out the paraffin block which contains the fixed culture.

3.6. Make 8 µm thick paraffin sections of the entire confronting pair using a microtome[14], and collect all sections on three alternating microscope glass slides that have been pretreated with tissue adhesive solution as a sticking agent. Avoid egg white as a sticking agent, because it may interfere with immunostaining.

Note: Pretreatment of the microscope glass slides is done by delivering 1 small drop of tissue adhesive solution and 3 small drops of demineralized water with a Pasteur pipet, and mixing them to cover the slide.

3.7. Remove the paraffin from the first slide by immersing twice in a jar containing 100 mL 100% xylene for 10 min.

3.8. Rehydrate the slides by immersion for 10 s in jars containing 100 mL of the following solutions: xylene/ethanol (1:1), 100% ethanol, 96% (v/v) ethanol in water, 70% (v/v) ethanol in water, and finally demineralized water.

3.9. Dissolve mercuric chloride crystals by immersion in a jar containing 100 mL of 0.5% (w/v) Iodine in 96% (v/v) ethanol in water for 2 min.

3.10. Clear the sections in a jar containing 100 mL of 5% (w/v) sodium thiosulfate in water for 10 sec. Then wash the slides thoroughly in distilled water.

3.11. Incubate the slides in a jar containing 100 mL Harris' hematoxylin for 2 min, submerge briefly in 0.1 M HCl, and then wash in running tap water for 10 min.

3.12. Incubate in a jar containing 100 mL eosin 0.1% (w/v) in water for 1 min.

3.13. Dehydrate via brief submersion in jars containing 100 mL of the following series of solutions: demineralized water, 70% (v/v) ethanol, 96% (v/v) ethanol, 100% ethanol twice, xylene–ethanol (1:1), and finally in 100% xylene.

3.14. Mount the slides with mounting medium by delivering 2 separate drops of the medium on the slides, let these expand by putting a coverslip on top of them, and let it harden at room temperature for 24 h.

4. Immunohistochemistry.

4.1. Prepare Tris-buffered saline (TBS). Dissolve 6.0 g of tris-(hydroxymethyl)-aminomethane (Tris base) and 45.0 g of NaCl in 4.5 L of demineralized water. Bring to pH 7.6 with 1 M HCl (about 42 mL). Add distilled water to make 5 L.

4.2. Prepare Tris-HCl buffer. Dissolve 60 g of Tris base in 800 mL of demineralized water. Bring to pH 7.6 with 6 M HCl (about 65 mL). Add distilled water to make 1 L. Dilute 10x with distilled water before use.

4.3. Prepare Tris-BSA 0.1% buffer. Dissolve 12.1 g of Tris base and 45.0 g of NaCl in 4.5 L of demineralized water. Bring to pH 8.2 with 1 M HCl (about 42 mL). Add 5.0 g of bovine serum albumin (BSA) and 6.5 g of NaN_3. Add demineralized water to make 5 L.

CAUTION: NaN3 is a highly toxic product. Contact with acids liberates very toxic gasses. Wear gloves and avoid ingestion by all means. NaN3 forms very sensitive explosive compounds with copper, lead and other metals. Flush sinks with copious amounts of water. An alternative preservative is 0.01% thiomersal.

4.4. Follow items 3.2 to 3.10.

4.5. Dip the slides in TBS or Tris–0.1% BSA buffer, and wipe off excess fluid around the sections using a paper towel.

4.6. Apply 150 µL normal goat serum diluted 1:20 in TBS or in 5% (w/v) BSA in Tris–0.1% (w/v) BSA buffer for 30 min in a high-humidity chamber (a closed box for the co-incubation

of the slides with an open water recipient ensuring high humidity inside). Then remove excess fluid with a paper towel.

4.7. Apply 150 µL primary antiserum diluted in Tris–0.1% (w/v) BSA supplemented with 1% (v/v) normal goat serum for at least 2 hr in a high-humidity chamber. Determine the optimal dilution of the primary antiserum empirically.

Note: Immunohistochemistry **c**an be used to detect chick heart antigens but, using specific antibodies, the technique described for chick heart antigens can be applied to other antigens (such as green fluorescent protein)[15] as well.

4.8. Wash the slides twice in Tris–0.1% w/v BSA on a rocking table for 5–10 min.

4.9. Remove excess fluid using a paper towel and apply 150 µL goat anti-rabbit antiserum in excess (e.g., 1:20 in TBS) for at least 1 hr in a high-humidity chamber.

4.10. Wash twice with TBS on a rocking table for 5–10 min.

4.11. Remove excess fluid using a paper towel. Apply the peroxidase–anti-peroxidase complex diluted 1:250 in TBS supplemented with 1% (v/v) normal goat serum for at least 1 hr in a high humidity chamber. The dilution of the complex depends on the batch.

4.12. Wash the slides with TBS and transfer to Tris–HCl buffer pH 7.6.

4.13. Transfer the slides into a Tris–HCl buffer containing 0.25 g/L of diaminobenzidine and 0.01% (v/v) H_2O_2 for a few minutes. Stop the incubation when the chick heart is stained. Check regularly under the microscope.

4.14. Transfer the slides to Tris–HCl for 10 min and then to distilled water.

4.15. Follow items 3.13 and 3.14.

Note: For antigens that are easily destroyed during fixation, cryosectioning of the cultures can offer an alternative for immunohistochemical analysis. This is described in the following protocol steps:

4.16. Wash the living cultures with 3 mL of Ringer's salt solution to remove serum proteins.

4.17. Add one drop of embedding medium to the precooled (−16 °C) specimen holder of a cryomicrotome. Transfer the cultured tissue from the edge of a glass coverslip to the top of this drop before it is completely frozen.

4.18. Cool the culture to −16 °C, cut 6 µm thick frozen sections, and collect them on gelatin-coated glass slides.

4.19. Fix the slides in acetone at 4 °C for 10 min before storage at 4 °C.

4.20. Follow items 4.5 to 4.15.

5. Evaluation of the assay results

Note: In the present assay, invasion is defined as the progressive occupation of the PHF by the confronting test cells. Microscopic analysis of all consecutive sections from a confronting culture allows the reconstruction of the interaction between the test cell aggregates and the PHFs in three dimensions.

5.1. Observe the different patterns of interaction and grade according to the following scale (see Figure 5).

Grade 0: Only PHF observed. No confronting cells can be observed.
Grade I: The confronting test cells are attached to the PHF, but do not occupy the heart tissue, not even the outermost fibroblastic cell layers.
Grade IIa: Occupation of the PHF is limited to the outer fibroblast-like and myoblast cell layers.
Grade IIb: The PHF has surrounded the cell aggregates but there are no signs of occupation.
Grade III: The confronting cells have occupied the PHF, but have left more than half of the original amount of heart tissue intact.
Grade IV: The confronting cells have occupied more than half of the original volume of the PHF.

Note: Grades I and II are observed with noninvasive cell populations, while grades III and IV are typical of invasion. To evaluate progression within different time frames, histological analysis should be applied to confronting cultures fixed after different incubation periods.

[Place Figure 5 here]

6. Toxicity assessment after drug treatments.

6.1. Transfer the confronting cultures in a minimal volume to 24-well tissue culture multidishes with a Pasteur pipet.

6.2. Wash the cultures by adding 500 μL of drug-free culture medium, and refresh after 6 hr.

6.3. Inspect all wells daily for cell outgrowth from the explants using an inverted microscope (objective lens 40X). Divide the number of outgrowing cultures by the total number of explanted cultures to obtain an index of survival of the confronting cultures. Compare the results of drug-treated cultures with solvent-treated ones.

Note: The use of Ki67 immunohistochemistry (for cell proliferation) and of TUNEL assay (for apoptosis) are suggested as complementary techniques to the explant assay for assessing toxicity.

7. Growth assessment of confronting cultures.

7.1. Make black-and-white photographs of the cultures just before fixation, using a microscope equipped with a digital photocamera (objective lens 50X)

7.2. Measure in mm the larger (**a**) and smaller (**b**) diameter of the culture taking into account the magnification.

7.3. Calculate the approximate volume (**V**) in mm^3 via the formula[16]
$V = 0.4 \times a \times b^2$

7.4. Calculate the growth of the culture by comparing this final volume with the combined volumes of PHF and cell aggregate at the start of the confrontation.

Note: Because solitary PHFs tend to decrease their volume during culture, growth of the confronting pairs is dependent on the tumor cells.

REPRESENTATIVE RESULTS:
The histological sections as presented in Figure 5 show the end result of a number of successful assays. The sound histology of the cultures indicates viable cells and allows to interpret the interaction between tumor cells and normal tissue. Furthermore, no immune reaction from the normal tissue can be observed, which confirms the correct age of the chick embryos used *e.g.* before the immune rejection system is developed. There are no bacteria visible, which indicates absence of (gross) contamination during the culture period. Finally the rounded periphery of the sections confirms culture in suspension without signs of (temporary) adherence to the Erlenmeyer flask wall.

Many compounds have been tested in the assay. Drugs are generally delivered to the culture medium at the moment when the confronting PHF/tumor aggregate pairs are transferred to small Erlenmeyer flasks (step 2.7). Some were used as tools (known inhibitors and activators) to unravel pathways and effector molecules implicated in the process of tumor invasion. For other compounds which were structurally related, a quantitative structure-activity relationship (QSAR) was established based on the results of the chick heart invasion assay. So, for related polyphenolics a number of computational descriptors were used to enable the prediction of their anti-invasive activity in the assay. Over a period of 15 years 139 different analogs were tested for their possible anti-invasive effects in the assay, and their activities were grouped into 4 classes. A training and a validation set consisting of 93 and 46 of those polyphenolics respectively were randomly selected. By means of a QSAR artificial neural network the results of the validation set showed a clear correlation between the predicted and experimental anti-invasive activities[17] (see Figure 6).

The data show the robustness of the chick heart invasion assay, since the correlation between predicted and experimental results was valid over 15 years, and confirmed in a recent (unpublished) prediction study with different polyphenolics. The confusion matrix presented in Figure 6 summarizes the weakness and the strength of the assay graphically: it gives a rough expression of the accuracy and the reproducibility. The interpretation of this graph should take into account the biological variability of living organ cultures, and the semi quantitative score of the invasion results.

[Place Figure 6 here]

Figure 1: Schematic overview of the different assay steps.

Figure 2: Lifting the embryo out of the egg with an enucleation spoon.

Figure 3: Aspiration of excess fluid around PHFs. Eight PHFs are placed on semi-solid agar in an embryological watch glass, and fluid excess is removed with a small triangular piece op filter paper. Arrows indicate the 8 individual PHFs.

Figure 4: Small Erlenmeyer flasks on top of a gyrotory shaker. The flasks with 1.5 mL of culture medium are sealed with silicone stoppers equipped with a gas in - and outlet needle, and moved at 120 rpm at 37 °C. The gas is led by an inlet tubing (1) to the larger Erlenmeyer flask (2) filled with sterile Ringer's salt solution, and further distributed (3 and 4) to smaller flasks containing culture medium with individual confronting pairs (5 and 6). Finally, spent gas is collected in a larger flask with a sterile water trap (7), from where it can escape into the air (8). The figure shows two sets of culture batteries on top of a home-made platform plate.

Figure 5: Examples of interactions between confronting cancer cells and PHFs. Histological sections of confronting cultures stained either with hematoxylin-eosin (left panels) or immunohistochemically with an antibody against chick heart (right panels). Interaction grades are defined in the protocol text. (H: heart tissue, T: tumor cells).

Figure 6: External validation of a predictive QSAR model (artificial neural network) for the activity of small molecules in the chick heart invasion assay. The output of this model is the anti-invasive activity class of a compound. Four such classes have been defined, representing the lowest concentration at which a molecule exerts anti-invasive activity (i.e. invasion grade I or II) in the CHI assay: class 4 (active down to 1 µM), class 3 (10 µM), class 2 (100 µM) and class 1 (no anti-invasive activity at concentrations as high as 100 µM). The depicted confusion matrix compares predicted and experimentally determined anti-invasive activity classes for the compounds of the validation set. The validation set contains 46 compounds, the training set 93. Model predictions are based solely on descriptors calculated from molecular structure, and can thus be obtained for hypothetical compounds. This way, synthetic efforts can be focused on molecules with promising *in silico* activity.

DISCUSSION:

During the preparation of PHFs, the fragments may not stay in suspension but adhere to the vessel wall; this can be overcome by increasing the volume of the culture medium. If the number of PHFs is too low and their size is too big, decrease the volume of the culture medium. Failure of the test cells to aggregate may be due to fluctuations in the temperature or to microbial infection. Alternatively, an inability to aggregate may be an intrinsic characteristic of the cells. During attachment of the aggregates to PHF, poor adhesion may be overcome by extending the incubation period on top of the semisolid agar medium or by removing more fluid culture medium around the cultures by means of absorbing filter paper. Check also for microbial contamination in this case. Difficulties during sectioning may be due to disintegration of the paraffin blocks: this occurs when the storage period of the blocks has been too long (melt the paraffin once again). When sectioning artifacts occur, the integrity of the microtome knife and the absence of *corpora aliena* in the fixed cultures should be checked. Necrotic areas in the cultures are signs of poor culture conditions. If these areas are restricted to the **center** of the cultures, one should suspect the volume of the confrontations being too large. **Proper selection of the volumes of the PHF and cell aggregates is indeed a critical factor.** However, more generalized necrosis points towards inappropriate pH control, microbial contamination or fixation artifacts. Finally, when the sections appear too dark, the immersion period in hematoxylin may be too long, or the sections may be too thick (>8 µm).

Many variations on the chick heart assay have been applied successfully in invasion studies. These variations relate to the origin of the host tissue, the presentation of the confronting test cells, and the incubation conditions. Heart fragments from species other than chick[18], and

from tissues such as liver[19], lung[20], and brain[21], have been examined. Instead of aggregates, biopsy specimens[22]monolayer fragments[23], and cell suspensions have been used to confront with PHF in organ culture. Suspension cultures are sometimes replaced by static cultures on top of a semisolid substrate[24], and serum-free confrontations have been shown to be feasible with certain types of cells[25]. Generally, the interaction is described in accordance with a semi quantitative scale[26] however, computer-assisted automated image analysis systems have also been developed[27,28]. The latter aim to provide quantitative information on the extent of tumor cell invasion.

As the chick heart assay includes a living host tissue, the setup attempts to recapitulate the situation *in vivo*, and clearly is of some relevance compared with other systems *in vitro* (see introduction section). It should, however, be recognized that the assay fails to encompass all the elements of the microecosystem present in natural tumors, environments where for example immunological factors can influence the invasive behavior of the cancer cells. In at least one study, the absence of such factors in the assay has led to conflicting results between the outcomes of the chick heart assay[29] and those of an animal model[30].

A future application will be the study of cardiomyocyte progenitor cells in the assay. These cells can be injected therapeutically into infarction zones of cardiac patients, but they should be able to integrate into the myocardium. In the chick heart assay the progenitor cells will be confronted with chick heart fragments, and their migration and differentiation will be analysed.

DISCLOSURES:
The authors declare that they have no competing financial interests.

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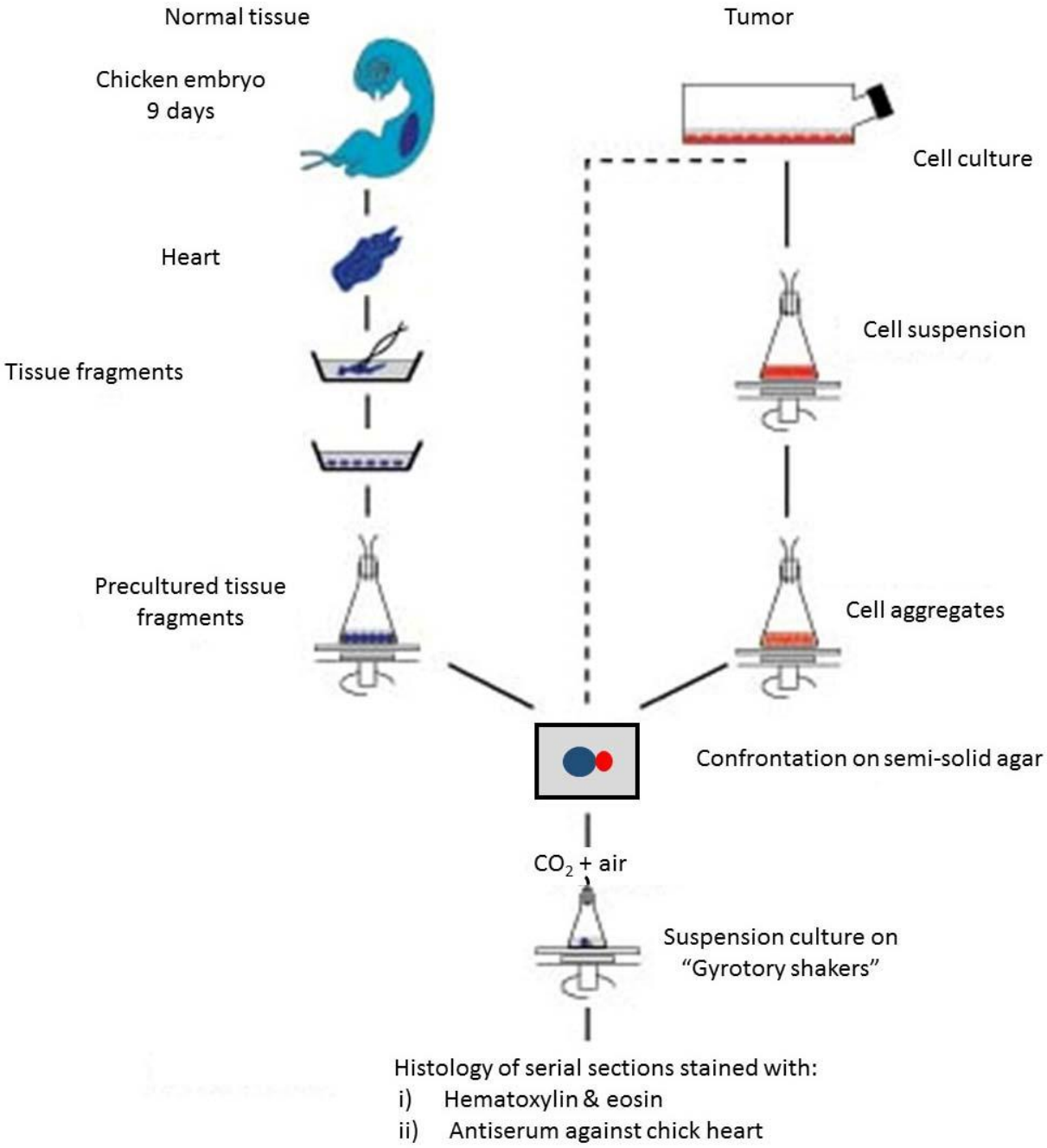

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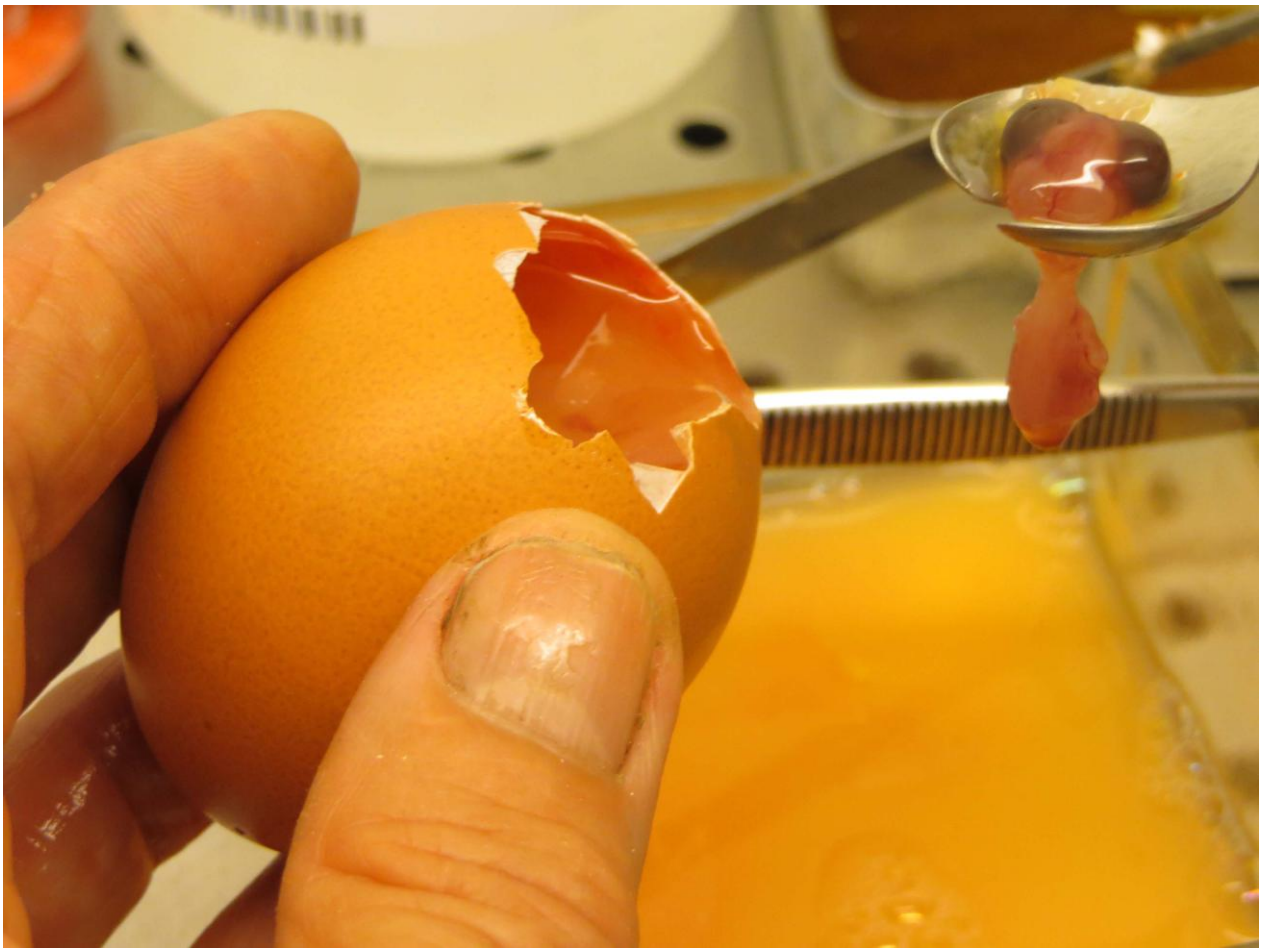

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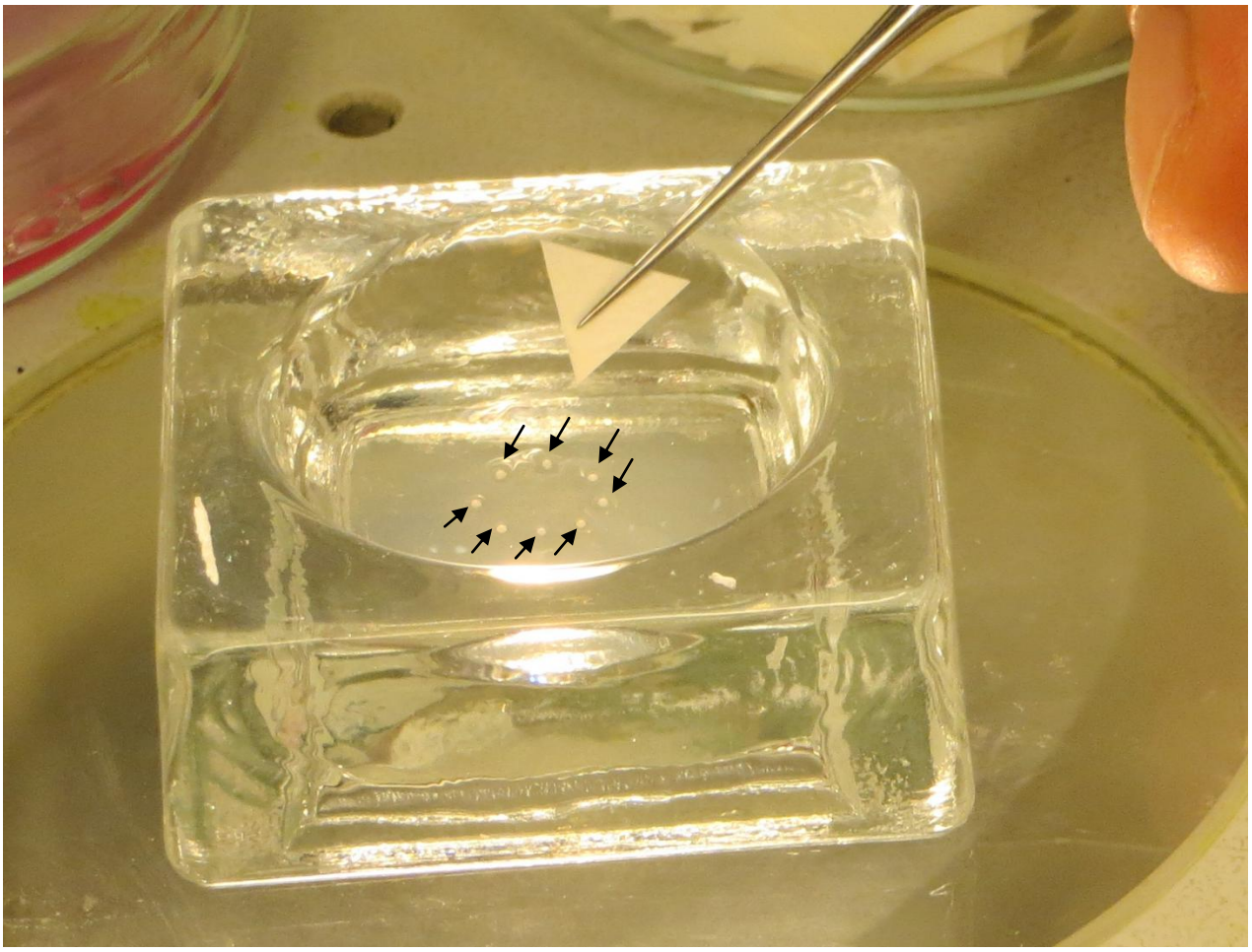

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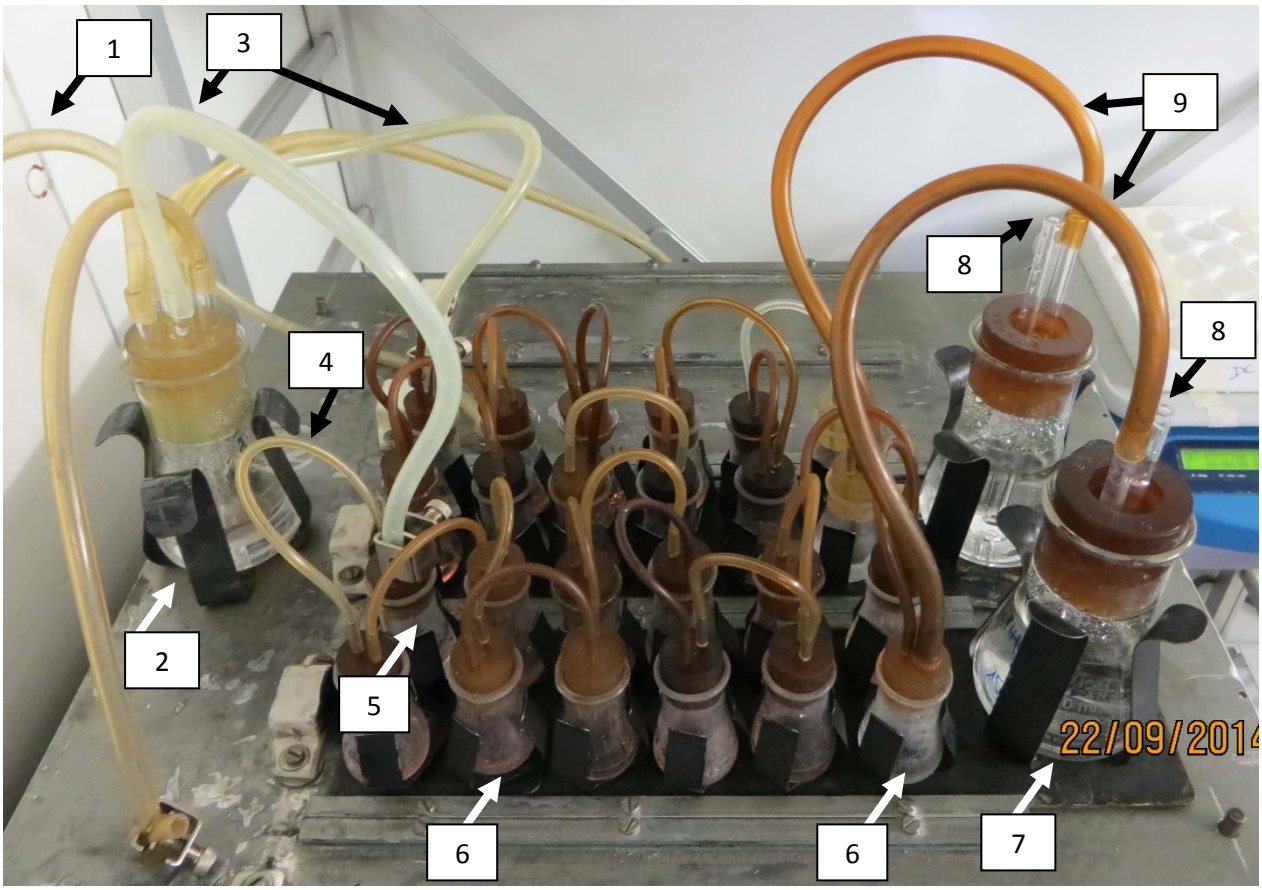

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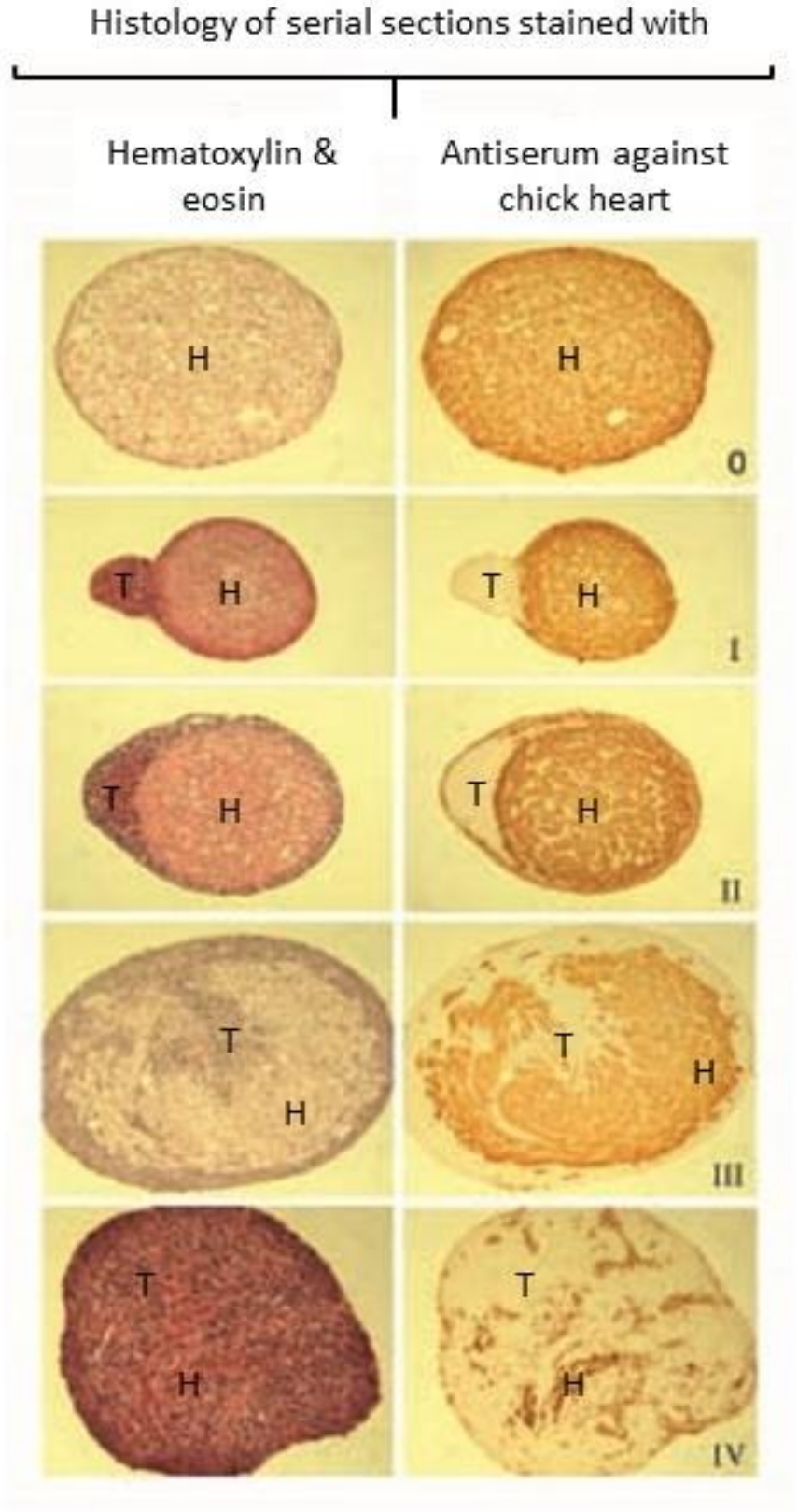

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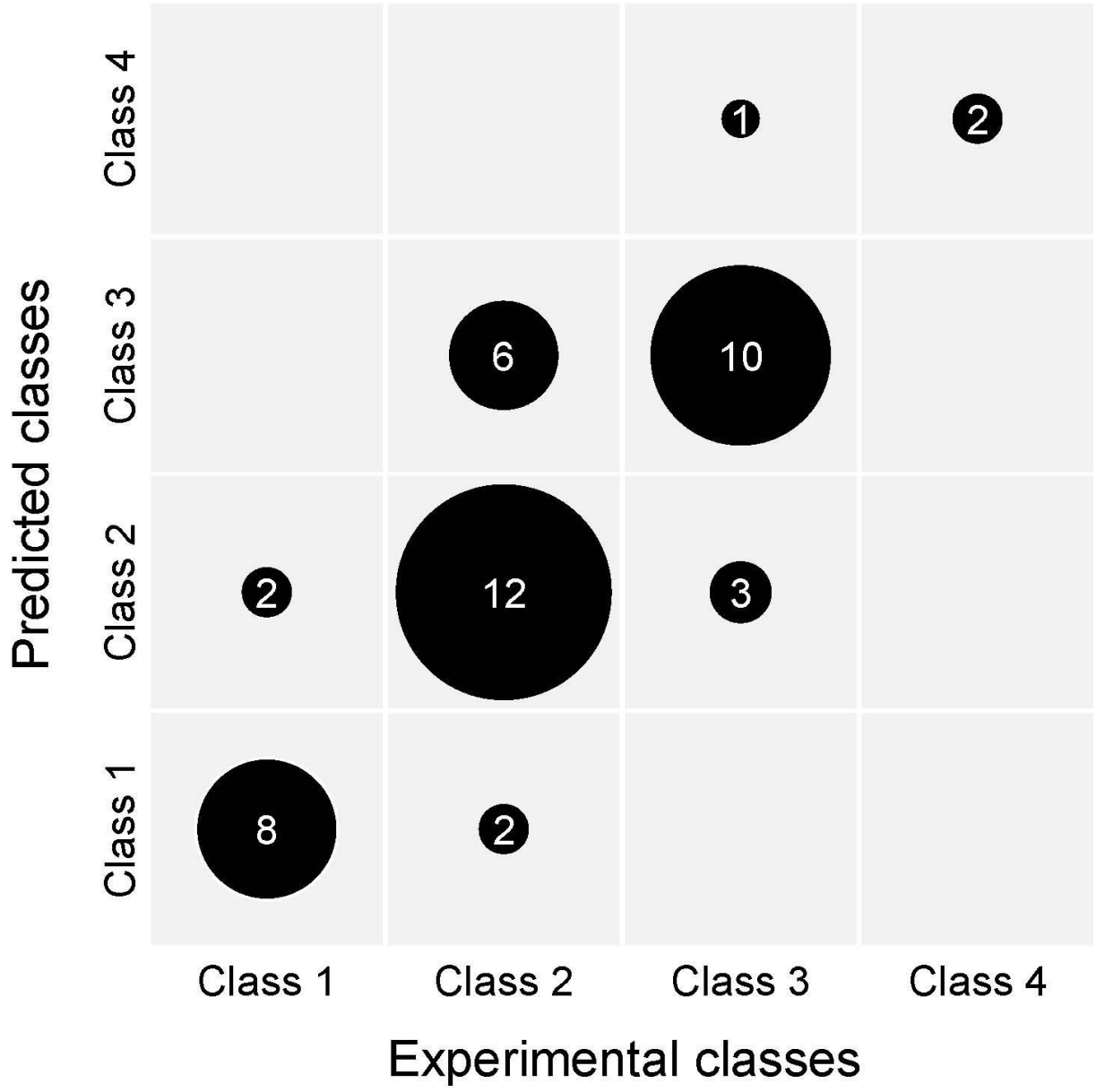

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Tris-(hydroxymethyl)-aminomethaan Analar	VWR	103157P
BSA : albumin from bovine serum, cohn V fract.	Sigma	A4503-500G
MEM-Rega 3	Life Technologies	19993013
Gyrotory shakers	New Brunswick Scientific	G10 and G33
Erlenmeyer flasks 50 ml	Novolab	92717
Glass Petri dishes	Novolab	68516
Iridectomy scissors	Rumex	11-0625
Macroscope with calibrated ocular grid	Wild	157702
Paraffin wax	International Medical products	8599956
Eosin Y	Sigma	230251-25g
Harris' hematoxylin	Sigma	HHS32-1L
Coverslipping resin (Tissue-Tek)	Sakura	4494
Paraffin melting apparatus	GFL	1052
Microtome for paraffin sectioning	Reichert	
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List of changes made by the authors in the revised manuscript JoVE52792R1

Changes in the revised manuscript (RM) are typed in green (underscored) or red.

Changes suggested by the Science Editor

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The authors followed this advice.

2. Throughout the text, dissect would sound better than "tear" for much of the animal work. On line 156 (RM) “Tear off” has been replaced by “Dissect”.

3. JoVE reference format requires that DOIs are included, when available, for all references listed in the article. The authors have done maximal efforts to obtain DOIs of the references. This was, however, not possible for a number of old references: even consultation of the original papers and books did not yield DOIs.

Changes suggested by the Reviewers.

Reviewer #1:

Protocol 1.1 (prep PHFs): Using a 9-day-old fertilized chick egg, "completing the incubation 4 days before the start…." is not clear and should be better explained. This sentence has been rephrased in the revised manuscript: “Complete this incubation by a date that allows subsequent preparation of PHFs during 4 days (*e.g.* on a Thursday,) and final confrontation with tumor cell aggregates (*e.g.* on the next Monday)

Protocol 1.4: Is the MEM-Rega 3 medium without FBS? The MEM-Rega 3 medium contains 5% FBS. This is specified in the revised manuscript.

Protocol 1.6: Is "fresh culture medium" meant to be the MEM-Rega 3 medium? With or without FBS? This medium is indeed MEM-Rega 3 medium containing 5% FBS. This is specified in the revised manuscript.

Figure 2: either arrows pointing towards or insets enlarging the "paired aggregates" should be useful, as they constitute only a minor part of the picture. In the revised figure the 8 PHFs are individually indicated by arrows.

Figure 3: arrows pointing towards the differently-sized Erlenmeyers and indicating their content, and arrows showing the CO2/air gas flow direction would be helpful. Is this device home-made? On the revised figure numbers are applied whose sequence indicates the gas flow in the suspension culture Erlenmeyer flasks. and are explained in the legend. The authors also specified in the revised figure legend that the device was home-made.

Protocol 6. Concerning the toxicity assessment: in addition to explant outgrowth, probably classical immunohistochemical tests for cell viability/proliferation and cell death (eg apoptosis) could be used? In a note added to section 6 the use of Ki67 immunohistochemistry

(for cell proliferation) and of TUNEL assay (for apoptosis) are suggested as complementary techniques to the explant assay for assessing toxicity.

Protocol 7. Concerning the growth assessment: the volume of the PHF does not increase but remains stable or decreases so one takes into account only the growth of the "confronting" cells? If so, this could be specified in the text. The comment of the reviewer is pertinent: during incubation solitary PHFs tend to decrease their volume. In a note added to section 7 the prominent impact of the confronting tumor cells on the growth of the confronting cultures is mentioned.

Figure 5 and corresponding text: the predictive QSAR model and its "classes" should be better explained or rephrased. In the revised manuscript the legend of this figure is rewritten to allow its interpretation independently from the main text:
"External validation of a predictive QSAR model (artificial neural network) for the activity of small molecules in the CHI assay. The output of this model is the anti-invasive activity class of a compound. Four such classes have been defined, representing the lowest concentration at which a molecule exerts anti-invasive activity (i.e. invasion grade I or II) in the CHI assay: class 4 (active down to 1 µM), class 3 (10 µM), class 2 (100 µM) and class 1 (no anti-invasive activity at concentrations as high as 100 µM). The depicted confusion matrix compares predicted and experimentally determined anti-invasive activity classes for the compounds of the validation set. The validation set contains 46 compounds, the training set 93. Model predictions are based solely on descriptors calculated from molecular structure, and can thus be obtained for hypothetical compounds. This way, synthetic efforts can be focused on molecules with promising in silico activity."

Reviewer #2:

The protocol description is not very clear at present. For example, the precise step where the heart fragments and cancer cells are mixed is not indicated at present. The authors could provide a flowchart of key steps. In the revised manuscript a schematic flowchart of the key assay steps is added as figure 1.

Models of organ culture tend to display large batch to batch variations. Some illustration of technical/biological reproducibility of assays will be useful. This comment of the reviewer is correct, and we believe that we addressed the reproducibility of the assay in the paragraph from line 479 to line 487 in the RM:
The data show the robustness of the chick heart invasion assay, since the correlation between predicted and experimental results was valid over 15 years, and confirmed in a recent (unpublished) prediction study with different polyphenolics. The confusion matrix presented in Figure 6 summarizes the weakness and the strength of the assay graphically: it gives a rough expression of the accuracy and the reproducibility. The interpretation of this graph should take into account the biological variability of living organ cultures, and the semi-quantitative score of the invasion results.
[Place Figure 6 here]

Figure 4 shows selected pictures representing different stages of the assay. If possible, this figure should include a pa. rallel panel of cells with different invasiveness (a non-invasive cell line, or the invasive cell line treated with a drug that inhibits its invasiveness). A demonstration of the utility of the technique presented here to capture "differences" in cell invasiveness is key to the potential utility of this platform for the wider community. We fear

that this comment is based on a misunderstanding. Figure 4 does not show the chronological progression of invasion by malignant tumor cells in the assay. Rather, it illustrates the final results obtained from cells with different invasiveness. These range from grades 0, I, II (coined non-invasion) to grades III,IV (invasion).

Reviewer #3:

There is a lack of figures actually showing how the heart is removed, fragmented and cultured to yield the host spheroids. We agree with the reviewer that the treatment of the heart can be better illustrated than described. However, actually we do not dispose of macrographs of the consecutive dissection steps, and, considering the limited revision time allotted, we were not able to organize a new experiment for making new pictures. We propose to take into account this reviewer's comment when it could come to video registration of this technique.

Histological figures presented are too small and should be marked with arrows to distinguish the key zones. Labels are missing to indicate what is the tumor being studied as this is a working example. In the revised manuscript the figure is enlarged, and heart or tumor zones are indicated with characters ("H" and "T" respectively)

Figure 5 is not informative without the additional information behind the analysis. In the RM the legend of this figure is rewritten to allow its interpretation independently from the main text:
"External validation of a predictive QSAR model (artificial neural network) for the activity of small molecules in the CHI assay. The output of this model is the anti-invasive activity class of a compound. Four such classes have been defined, representing the lowest concentration at which a molecule exerts anti-invasive activity (i.e. invasion grade I or II) in the CHI assay: class 4 (active down to 1 µM), class 3 (10 µM), class 2 (100 µM) and class 1 (no anti-invasive activity at concentrations as high as 100 µM). The depicted confusion matrix compares predicted and experimentally determined anti-invasive activity classes for the compounds of the validation set. The validation set contains 46 compounds, the training set 93. Model predictions are based solely on descriptors calculated from molecular structure, and can thus be obtained for hypothetical compounds. This way, synthetic efforts can be focused on molecules with promising in silico activity."

What is bothersome in the protocol is the use of toxic reagents both in the fixative and antibody preservation. There are good substitutes, and the commercial firms have moved beyond use of sodium azide. The authors should offer non-toxic alternatives that work just as well. In the "caution" paragraph concerning the fixation procedure (lines 258 to 259) an alternative procedure is mentioned with 4% formaldehyde in phosphate –buffered saline. In the "caution" paragraph concerning azide (line 357) the use of 0.01% thiomersal is mentioned as an alternative preservative.

1.7 'ventricular'-typo. This is corrected in the reversed version.

1.9 What is the rationale for this step? The rationale for this suspension culture step is to obtain spheroidal heart tissue fragments suitable for subsequent confrontation with tumor cell aggregates. This rationale is added to the revised manuscript.

Reviewer #4:

This experiment has been done on heart tissue that was stripped from its pericardium. What is the purpose of this step? Doesn't it diminish the significance of this assay as opposed to what happen in some types of lung or esophageal cancers that directly invade the heart along with its pericardium? The comment is considered as correct by the authors. So, the assay may indeed not be a relevant model for heart invasion by lung or esophageal cancers. For practical reasons, however, we prepare precultured heart fragments (PHFs) surrounded by fibroblastic cells, since the initial amount of pericardial mesothelium is theoretically not sufficiently present to literally cover homogeneously all PHFs needed for an experiment. The mesothelium-free myocardium-rich PHFs are meant as a general substrate to assess invasion of multiple cell types.

In the text, it is mentioned that the same type of assay has been described before but from heart fragments taken from other species, or using other organs. What are the advantages or disadvantages of this particular organ? And the species? It should be discussed in the text. The choice of heart over other organ fragments was based on the spontaneous rhythmic pulsations of many of the PHFs. These pulsations are used as indicators of possible heart toxicity of test drugs in the culture medium. The authors preferred avian embryos, because they can easily be dissected from the sterile content of the egg. The rationale these choices are added to the discussion section of the RM (lines 112 to 115).

It should be described for the reader how a particular inhibitor of invasion is added to this assay? Drugs are generally delivered to the culture medium at the moment when the confronting PHF/tumor aggregate pairs are transferred to small Erlenmeyer flasks (step 2.7). This information is added in lines 465 to 478 in the RM.

The rate of variability of this technique should also be mentioned especially that it is a laborious technique that would require several days (30) to perform. This comment of the reviewer is correct, and we believe that we addressed the reproducibility of the assay in the paragraph from line 479 to line 487:
The data show the robustness of the chick heart invasion assay, since the correlation between predicted and experimental results was valid over 15 years, and confirmed in a recent (unpublished) prediction study with different polyphenolics. The confusion matrix presented in Figure 6 summarizes the weakness and the strength of the assay graphically: it gives a rough expression of the accuracy and the reproducibility. The interpretation of this graph should take into account the biological variability of living organ cultures, and the semi-quantitative score of the invasion results.
[Place Figure 6 here]