

SUMMARY OF THE INVENTION

In one aspect of the present disclosure, there is provided a method to establish tumors from primary tumor cells obtained from biopsies or surgically removed tumors comprising:

- a. Isolating one or more primary tumor cells;
- b. Labeling the tumor cells with a cell tracking dye;
- c. Injecting the tumor cells into a 24 to 48 hours post fertilization (hpf) zebrafish embryo; and
- d. Incubating the embryo for 24 hours or more.

In another aspect of the present disclosure, there is provided a multi-well microinjection system automating microinjection of 24 to 72 hpf zebrafish embryos comprising:

- a. a holding frame; a plurality of removable multi-well modules, wherein each multi-well module consists of a groove plate and a removable insert; each groove plate has a plurality of embryo holding wells having conical open bottoms which are arranged in a linear format; a well module at an outer edge of the groove plate has a cylindrical shape, thus permitting liquid handling through the well module; a removable insert, having vertical sides and upper circular openings which are aligned with each groove in the groove plate, thus forming an embryo holding and handling well when placed over the top of the groove plate; and a lid for covering the holding frame, the groove plate and removable insert.
and
- b. microinjection micropipette rotatably positioned over the multi-well plate for enabling injecting the embryo at variable angles and/or height.

In a further aspect of the present disclosure, there is provided a method for testing a drug for its effect on tumor cells, comprising:

- a. microinjecting one or more tumor cells into a 24 to 72 hour post-fertilization zebrafish embryo;
- b. allowing the tumor cells to grow within the zebrafish embryo for a predetermined time;
- c. incubating or microinjecting the zebrafish embryo with a drug; and

- d. monitoring the effect of the drug on the tumor cells by measuring the tumor cells in the embryo after the pre-determined time.

AIMS OF THE INVENTION-PRESENT DISCLOSURE

The aims of the present ~~invention-disclosure~~ were to overcome the technical problems of profiling and characterizing primary tumor cells and CTC in an animal model, and to provide a system that would enable efficient manipulation and injection of zebrafish embryos.

The applicant has discovered that these technical problems are solved by way of methods that include creating viable tumors from xeno -transplanted human CTCs into zebrafish, studying the metastatic potential of the injected CTCs, predicting the organ preference of isolated CTCs, and assessing the response to therapeutic agents. A high-throughput accelerated assay method of predicting cancer progression and response to chemotherapy is also presented.

STATEMENTS OF INVENTION-~~THE~~ PRESENT DISCLOSURE

In one aspect of the invention, a method is provided to develop three-dimensional tumors from primary tumor cells obtained from biopsies or surgically removed tumors by steps comprising:

- (a) Isolating tumor cells
- (b) Labeling tumor cells with a cell tracking dye
- (c) Injecting the cells into a 24 to 48 hours post fertilization (hpf) zebrafish embryo
- (d) Incubating the embryos for 24 hours or more

In one embodiment, the tumor cells are obtained from circulating tumor cells (CTCs).

In another embodiment, the cell tracking dye is a fluorescent dye.

CLAIMS

What is claimed is:

1. A method to establish tumors from primary tumor cells obtained from biopsies or surgically removed tumors comprising:
 - 5 (a) Isolating primary tumor cells
 - (b) Labeling the tumor cells with a cell tracking dye
 - (c) Injecting the cells into a 24 to 48 hours post fertilization (hpf) zebrafish embryo
 - (d) Incubating the embryos for 24 hours or more.
2. The method of claim 1 or claim 2, wherein the tumor cells are obtained from circulating
10 tumor cells (CTCs).
3. The method of claim 1, wherein the cell tracking dye is a fluorescent dye.
4. A method to predict the likelihood of a primary tumor to invade or metastasize comprising:
 - (a) Injecting the tumor cells into the yolk of a zebrafish embryo
 - (b) Incubating the embryos injected with the tumor cells
 - 15 (c) Observing the position of the tumor cells after incubation
 - (d) Analyzing whether the tumor cells (i) invade the body of the embryo through the yolk sac or (ii) whether the cells remain in the yolk sac and behave closely to non-invasive primary tumor cells.
5. A method to identify cancer patients with a higher probability of disease relapse comprising:
 - 20 (a) Injecting the primary tumor cells into the yolk of the embryo
 - (b) Incubating the embryos with the injected tumor cells
 - (c) Observing the position of the tumor cells after incubation

(d) If the tumor cells enter the body of the embryo, the propensity of the primary tumor to invade and metastasize is high.

6. A method to quantitate cancer cell invasion comprising:

(a) Isolating tumor cells from a solid tumor

5 (b) Labeling tumor cells with a cell tracking dye

(c) Micro-injecting the cells into the yolk of a 24-48hpf zebrafish embryo

(d) Incubating the embryos at 35degC for 24h or more

(e) Capturing automated fluorescent images of the tumor cells under a fluorescent microscope

10 (f) Automated analysis of the tumor foci using image analysis software to capture the Width (W) and Length (L) of the tumor foci, the intensity of the signal on each foci, and the position of the spot in the image

(g) Calculating the volume of the foci as $1/2WL^2$, where W=Width and L=Length.

15 (h) Using the position of the tumor foci to measure the propensity of the primary tumor to invade. Invasive Index can be measured as: Invasion Index (II) = $1/n \sum (\text{number of tumor foci in the embryo at T hours} / \text{Total number of tumor cells injected in the embryo})$, where n is the number of embryos considered in the experiment, and T is the incubation time T, and the greater the II, the higher the propensity of the primary tumor to invade.

20 (i) Using the position of the tumor foci to measure the invasive aggressiveness, using the Migration Index, calculated as Migration Index (MI) = $1/n \sum (\text{CD at T hours} / \text{Total number of tumor foci at time T hours})$, where CD=Cumulative distance traveled by tumor cells, n is the number of embryos considered in the experiment, and T is the incubation

time, where the higher the value of the MI, the more aggressively invasive is the tumor cells.

7. A method as in claim 6, wherein the response to a chemical by the tumor cells is measured by determining whether any one of: the volume of the tumor foci; the Invasion Index; or the Migration Index are different in the presence versus the absence of said chemical.

8. A method to predict the preferred organ for homing of the tumor cells, comprising:

(a) Isolating tumor cells from a solid tumor

(b) Labeling cells with a cell tracking dye that has a red fluorescence, such as but not limited to, PKH-26 or DiD

(c) Micro-injecting the cells into the yolk of a 24-48 hpf Tg(Fli:EGFP) zebrafish embryo

(d) Incubating the embryos at 35degC for 24h or more

(e) Capturing automated fluorescent images of the tumor cells under a fluorescent microscope using filters for both green and red fluorescence

(f) Automated analysis of the tumor foci using image analysis software, to capture the position of the foci in the image.

(g) Use the image analysis to calculate the Homing Index of the tumor as: Homing Index (HI) = $1/n \sum (\text{Total number of foci in an organ at T hours} / \text{Total number of tumor foci at time T hours})$, where n is the number of embryos considered in the experiment, and T is the incubation time T.

9. A method of monitoring changes in the zebrafish immune system during tumor invasion, metastasis or organ homing processes comprising:

(a) Using genetically modified embryos with fluorescent proteins expressed in immune cells, such as but not limited to zebrafish embryos expressing gata2-GFP, to monitor

localization and changes in the number of specific immune cells, such as but not limited to eosinophils, present at any part of the zebrafish body.

(b) Using whole embryo immuno-histochemical staining of embryos to locate and enumerate immune cells.

5 10. A method for measuring the number of surviving tumor cells after incubation with or without synthesized or naturally occurring chemicals or biologicals is provided comprising:

(a) Digesting zebrafish embryos in a protease solution

(b) Dispersing cells gently dispersed with pipetting to dissociate the zebrafish embryo to a single cell suspension

10 (c) Fixing and counting cells under a fluorescent microscope

(d) Comparing the ratio of the total number of viable fluorescent tumor cells to the injected number of cells between treated and untreated zebrafish embryos to predict the effect of synthesized or naturally occurring chemicals and biologicals versus untreated embryos

11. A method is provided to predict drug efficacy against tumor cell invasiveness, comprising
15 measuring and comparing the patterns of invasiveness of tumor cells in the presence or absence of the drug and comparing whether cell invasiveness is different in the presence of the drug.

12. A method to predict a drug effect on the organ homing preference of cancer cells comprising
20 observing the organ-homing pattern change of the injected tumor cells in the absence versus the presence of said drug.

13. A method for assessing changes in the DNA of tumor cells comprising:

a. Enzymatic digestion of whole embryo or part of zebrafish tissues that contain tumor cells

b. Isolation of DNA from the digested embryo or tissues

- c. PCR amplification of genes of interest using PCR primers designed for human genetic sequences.
- d. Sequencing to identify genetic mutations
- e. Bisulfite sequencing to locate epigenetic modifications

5 14. A method to analyze gene expression in cancer cells comprising:

- (a) Enzymatic digestion of whole embryo or part of zebrafish tissues that contain tumor cells
- (b) Isolation of RNA from the digested embryo or tissues
- (c) Quantitative Real-Time PCR analysis of gene expression using specific primers designed for human genetic sequences.

10 15. A method to analyze protein expression in cancer cells comprising one of:

- (a) Fixing the whole embryo using a chemical fixative such as 4% paraformaldehyde and visualizing protein expression using immunohistochemistry with specific antibodies to human proteins;
- (b) Visualizing proteins using immunohistochemistry on histological section slides of the
15 zebrafish embryo after injection with tumor cells
- (c) Visualizing protein expression using ELISA (enzyme-linked immunosorption assay)
or
- (d) Visualizing protein expression using Western blot.

20 16. A multi-well microinjection system automating microinjection of 24 to 72 hpf zebrafish embryos comprising:

- (A) a holding frame; a plurality of removable multi-well modules, wherein each multi-well module consists of a groove plate and a removable insert; each groove plate has a plurality of embryo holding wells having conical open bottoms which are arranged in a

linear format; a well module at an outer edge of the groove plate has a cylindrical shape,us permitting liquid handling through this well; a removable insert, having vertical sides and upper circular openings which are aligned with each groove in the groove plate, thus forming an embryo holding and handling well when placed over the top of the groove plate; and a lid for covering the holding frame, the groove plate and removable insert.

and

(B) microinjection micropipette rotatably positioned over the multi-well plate for enabling injecting the embryo at variable angles and/or height.

17. The multi-well microinjection system of claim 16, wherein the plurality of embryo holding wells are each interconnected at their open conical bottoms to the open bottom of the well module at an outer edge of the groove plate

18. The multi-well microinjection system of claim 16 or claim 17, wherein the automation of the microinjection is performed using a robotic arm controlled micropipette holder.

19. The multi-well microinjection system of any one of claims 16 to 18, inclusive, wherein the automation of the microinjection is performed using a micropipette which is controlled by a robotic arm.

20. The multi-well microinjection system of claim 16 or 17, wherein the micropipette injection system is structured and arranged to be rotatably positionable, thereby to inject the embryo at variable angles and/or heights.

21. The multi-well microinjection system of any one of claims 16 to 20, inclusive, wherein the position and and/or angle of the robotic arm is adjustable, either manually or according to a software control interface.

22. The multi-well microinjection system of claim 21, wherein the software control interface is a commercially-available microinjector injection system, or through the development of a specific automatic injection system which is specifically designed for microinjection into zebrafish embryos.

23. The multi-well microinjection system of claim 21, wherein the robotic arm is controlled through human vision recognition of embryo structures or vision recognition of embryo structures by means of fluorescence labelling of the cells, or by means of software which is programmed to enable detection of the success of injection of liquids, the software being either a commercially-available microinjector injection system, or through the development of a specific automatic injection system which is specifically designed for microinjection into zebrafish embryos.

24. The multi-well microinjection system of any one of claims 21 to 23, inclusive, wherein automation for the selection of the sites of injection and the protocols of injection is changed by software updates.

25. A method for the automated microinjection of 24 to 72 hour post-fertilization zebrafish embryos, comprising:

(a) placing a plurality of 24 to 72 hour post-fertilization zebrafish embryos in associated ones of multi-well modules of the multi-well microinjection system of any one of claims 16 to 24, inclusive; and

(b) microinjecting a selected molecule into the yolk of the zebrafish embryo.

26. A method for the automated microinjection of 24 to 72 hour post-fertilization zebrafish embryos of claim 25, wherein the selected molecule comprises tumor cells.

27. The method for the automated microinjection of 24 to 72 hour post-fertilization zebrafish embryos of claim 26 including the addition either before, during or after the microinjection of the tumor cells, of a pro-angiogenic factor into the water containing the zebrafish embryo.
28. The method for the automated microinjection of 24 to 72 hour post-fertilization zebrafish embryos of claim 27, wherein the pro-angiogenic factor is growth factor angiopoietin.
29. A method for causing tumor cells to be efficiently taken up by the embryo of a zebrafish, comprising microinjecting tumor cells into the yolk of the 24 to 72 hour post fertilization zebrafish embryo and either before, during or after the microinjection of the tumor cells, the addition of a pro-angiogenic factor into the water containing the zebrafish embryo.
30. The method for causing tumor cells to be efficiently taken up by the embryo of a zebrafish of claim 29, wherein the pro-angiogenic factor is growth factor angiopoietin.
31. A method for testing drugs for their effect on tumor cells, comprising:
- (a) microinjecting tumor cells into the embryo of a 24 to 72 hour post-fertilization zebrafish embryo;
 - (b) allowing the tumor to grow within that zebrafish embryo for a predetermined time;
 - (c) incubating the embryo in system water with the drug or microinjecting the drug being tested for its effect on tumor cells into that 24 to 72 hour post-fertilization zebrafish embryo; and
 - (d) monitoring the effect of that drug on the tumor cells by measuring the amount of tumor cells in the 24 to 72 hour post fertilization zebrafish embryo.
32. The method for testing drugs for their effect on tumor cells of claim 31 in 24 to 72 hour post fertilization zebrafish embryos, and either before, during or after the microinjection of the

tumor cells, the addition of a pro-angiogenic factor into the water containing the zebrafish embryo.

33. The method for testing drugs for their effect on tumor cells of claim 32, wherein the pro-angiogenic factor is growth factor angiopoietin.

5 34. The method for testing drugs for their effect on tumor cells of any of claims 27, 29, or 32, in which the pro-angiogenic factor is microinjected into the 24 to 72 hour post fertilization zebrafish embryo.

CLAIMS

What is claimed is:

1. A method to establish tumors from primary tumor cells obtained from biopsies or surgically removed tumors comprising:
 - a. Isolating one or more primary tumor cells;
 - b. Labeling the tumor cells with a cell tracking dye;
 - c. Injecting the tumor cells into a 24 to 48 hours post fertilization (hpf) zebrafish embryo; and
 - d. Incubating the embryo for 24 hours or more.
2. The method of claim 1, wherein the tumor cells are circulating tumor cells.
3. The method of claim 1, wherein the cell tracking dye is a fluorescent dye.
4. The method of claim 1, wherein the tumor cells are injected into a 24 to 48 hpf zebrafish embryo yolk.
5. The method of claim 4, further comprising measuring the tumor cells' position after incubating the embryo to determine whether the tumor cells (i) invade the embryo body; or (ii) remain in the yolk sac.
6. The method of claim 5, further comprising using the tumor cells' position to indicate tumor cell metastatic potential, wherein if the tumor cells invade the embryo body, the tumor cells have metastatic potential.
7. The method of claim 5, wherein the tumor cells' position is measured by capturing one or more fluorescence images of the tumor cells under a fluorescent microscope.
8. The method of claim 7, further comprising quantitating the tumor cells in the embryo yolk sac and the embryo body by one or more of:

- a. using image analysis software to measure a Width and a Length of the tumor cells' foci and calculating a Volume of the tumor cells' foci as $\frac{1}{2}(\text{Width})(\text{Length})^2$;
 - b. measuring an Invasive Index of the tumor cells' foci as Invasive Index (II) = $\frac{1}{n} \sum (\text{number of tumor foci in the embryo at T hours} / \text{Total number of tumor cells injected in the embryo})$, where n is the number of embryos considered in the experiment, and T is the incubation time, and the greater the II, the higher the propensity of the primary tumor to invade; and
 - c. measuring a Migration Index of the tumor cells as Migration Index (MI) = $\frac{1}{n} \sum (\text{CD at T hours} / \text{Total number of tumor cells foci at time T hours})$, where CD=Cumulative distance traveled by tumor cells, n is the number of embryos considered in the experiment, and T is the incubation time, where the higher the value of the MI, the more aggressively invasive are the tumor cells.
9. The method of claim 8, further comprising determining whether any one of: the Volume of the tumor cells' foci; the Invasive Index; or the Migration Index are different in the presence versus the absence of a chemical.
 10. The method of claim 1, wherein the embryo incubation is in the presence or the absence of a chemical.
 11. The method of claim 10, further comprising measuring an effect of the chemical on the tumor cells by:
 - a. Digesting the embryo and the tumor cells in a protease solution after incubation;
 - b. Dispersing the embryo and the tumor cells with pipetting to dissociate to a single cell suspension;

- c. Fixing and counting the tumor cells in the single cell suspension under a fluorescent microscope;
 - d. Calculating a ratio of the counted tumor cells to the injected tumor cells;
 - e. Comparing the ratio for incubation in the presence of the chemical to the ratio for incubation in the absence of the chemical; and
 - f. Using the comparison to determine whether the chemical affects the tumor cells.
12. The method of claim 10, further comprising measuring and comparing the patterns of invasiveness of tumor cells in the presence or absence of the chemical.
13. The method of claim 1, further comprising assessing changes in the tumor cells' DNA by:
- a. enzymatically digesting the embryo and the tumor cells after incubation;
 - b. isolating the digested embryo and tumor cells DNA;
 - c. PCR amplifying one or more genes from the digested embryo and tumor cells DNA;
 - d. Sequencing the amplified one or more genes; and
 - e. Bisulfite sequencing the digested embryo and tumor cells DNA to locate epigenetic modifications.
14. The method of claim 1, further comprising analyzing tumor cells' gene expression by:
- a. Enzymatically digesting the embryo and the tumor cells after incubation;
 - b. Isolating the digested embryo and the tumor cells' RNA;
 - c. Performing a Quantitative Real-Time PCR analysis of gene expression using two or more primers designed for human genetic sequences.
15. The method of claim 1, further comprising analyzing tumor cells' protein expression by one or more of:

- a. Fixing the embryo using a chemical fixative and using immunohistochemistry with one or more human protein antibodies to visualize tumor cells' protein expression;
 - b. Visualizing tumor cells' protein expression using immunohistochemistry on histological section slides of the embryo after tumor cell injection and incubation;
 - c. Visualizing tumor cells' protein expression using ELISA (enzyme-linked immunosorption assay); and
 - d. Visualizing tumor cells' protein expression using Western blot.
16. The method of claim 1, wherein a pro-angiogenic factor is added into water containing the zebrafish embryo before, during or after the microinjection of the tumor cells
17. The method of claim 16, wherein the pro-angiogenic factor is angiopoietin.
18. The method of claim 1, further comprising:
- a. the cell tracking dye has a red fluorescence;
 - b. the tumor cells are injected into a 24 to 48 hpf Green Fluorescent Protein transgenic zebrafish embryo yolk;
 - c. capturing one or more fluorescent images of the tumor cells under a fluorescent microscope using green and red fluorescence filters after incubating the embryo;
 - d. analyzing the fluorescent images using an image analysis software to capture the tumor cells' foci position in the image;
 - e. using the analysis of the fluorescent images to calculate a Homing Index of the tumor cells as: Homing Index (HI) = $1/n \sum (\text{Total number of tumor cells foci in an organ at T hours} / \text{Total number of tumor cells foci at time T hours})$, where n is the number of embryos considered in the experiment, and T is the incubation time T.

19. The method of claim 18, further comprising observing the organ-homing pattern change of the injected tumor cells in the absence versus the presence of a drug.
20. The method of claim 18, wherein the tumor cells are circulating tumor cells.
21. A multi-well microinjection system automating microinjection of 24 to 72 hpf zebrafish embryos comprising:
 - a. a holding frame; a plurality of removable multi-well modules, wherein each multi-well module consists of a groove plate and a removable insert; each groove plate has a plurality of embryo holding wells having conical open bottoms which are arranged in a linear format; a well module at an outer edge of the groove plate has a cylindrical shape, thus permitting liquid handling through the well module; a removable insert, having vertical sides and upper circular openings which are aligned with each groove in the groove plate, thus forming an embryo holding and handling well when placed over the top of the groove plate; and a lid for covering the holding frame, the groove plate and removable insert.and
 - b. microinjection micropipette rotatably positioned over the multi-well plate for enabling injecting the embryo at variable angles and/or height.
22. The multi-well microinjection system of claim 21, wherein the plurality of embryo holding wells are each interconnected at their open conical bottoms to the open bottom of the well module at an outer edge of the groove plate.
23. The multi-well microinjection system of claim 21, wherein the automation of the microinjection is performed using a micropipette which is controlled by a robotic arm.

24. The multi-well microinjection system of claim 21, wherein the micropipette injection system is structured and arranged to be rotatably positionable, thereby to inject the embryo at variable angles and/or heights.
25. The multi-well microinjection system of claim 21, wherein the position and/or angle of the robotic arm is adjustable, either manually or according to a software control interface.
26. A method for testing a drug for its effect on tumor cells, comprising:
 - a. microinjecting one or more tumor cells into a 24 to 72 hour post-fertilization zebrafish embryo;
 - b. allowing the tumor cells to grow within the zebrafish embryo for a predetermined time;
 - c. incubating or microinjecting the zebrafish embryo with a drug; and
 - d. monitoring the effect of the drug on the tumor cells by measuring the tumor cells in the embryo after the pre-determined time.
27. The method of claim 26, wherein the tumor cells are circulating tumor cells.
28. The method of claim 26, further comprising the addition of a pro-angiogenic factor into a water containing the zebrafish embryo either before, during or after the microinjection of the tumor cells.
29. The method of claim 28, wherein the pro-angiogenic factor is angiopoietin.
30. The method of claim 28, wherein the pro-angiogenic factor is microinjected into the embryo.