

SUMMARY

In one aspect of the present disclosure, there is provided a method ~~to establish tumors from primary tumor cells~~ for analysis of circulating tumor cells (CTCs) obtained from biopsies or surgically removed tumors comprising:

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

In another aspect of the present disclosure, there is provided a multi-well microinjection system ~~automating microinjection of 24 to 72 hpf zebrafish embryos~~ to inject the circulating tumor cells as disclosed in the methods disclosed herein 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 circulating tumor cells, comprising:

- a. microinjecting one or more circulating tumor cells into a 24 to 72 hour post-fertilization zebrafish embryo;
- b. allowing the circulating 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 circulating tumor cells by measuring the circulating tumor cells in the embryo after the pre-determined time.

AIMS OF THE PRESENT DISCLOSURE

The aims of the present 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 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~~ for analysis of circulating tumor cells (CTCs) obtained from biopsies or surgically removed tumors comprising:
 - a. Isolating one or more ~~primary~~ circulating tumor cells;
 - b. Labeling the circulating tumor cells with a cell tracking dye;
 - c. Injecting the circulating tumor cells into a 24 to 48 hours post fertilization (hpf) zebrafish embryo; ~~and~~
 - d. Incubating the embryo for 24 hours or more; and
 - e. Analyzing the circulating tumor cells in the zebrafish embryo after incubation.
- ~~2. The method of claim 1, wherein the tumor cells are circulating tumor cells.~~
- ~~3~~2. The method of claim 1, wherein the cell tracking dye is a fluorescent dye.
- ~~4~~3. The method of claim 1, wherein the circulating tumor cells are injected into a 24 to 48 hpf zebrafish embryo yolk.
- ~~5~~4. The method of claim ~~4~~3, further comprising measuring the circulating tumor cells' position after incubating the embryo to determine whether the circulating tumor cells (i) invade the embryo body; or (ii) remain in the yolk sac.
- ~~6~~5. The method of claim ~~5~~4, further comprising using the circulating tumor cells' position to indicate circulating tumor cell metastatic potential, wherein if the circulating tumor cells invade the embryo body, the circulating tumor cells have metastatic potential.
- ~~7~~6. The method of claim ~~6~~5, wherein the circulating tumor cells' position is measured by capturing one or more fluorescence images of the circulating tumor cells under a fluorescent microscope.
- ~~8~~7. The method of claim ~~7~~6, further comprising quantitating the circulating 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 circulating tumor cells' foci and calculating a Volume of the circulating tumor cells' foci as $\frac{1}{2}(\text{Width})(\text{Length})^2$;
- b. measuring an Invasive Index of the circulating tumor cells' foci as Invasive Index (II) = $\frac{1}{n} \sum (\text{number of tumor foci in the embryo at T hours} / \text{Total number of } \underline{\text{circulating}} \text{ tumor cells injected in the embryo})$, 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~~ circulating tumor cells to invade; and
- c. measuring a Migration Index of the circulating tumor cells as Migration Index (MI) = $\frac{1}{n} \sum (\text{CD at T hours} / \text{Total number of } \underline{\text{circulating}} \text{ tumor cells foci at time T hours})$, where CD=Cumulative distance traveled by circulating 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 circulating tumor cells.

98. The method of claim 87, further comprising determining whether any one of: the Volume of the circulating tumor cells' foci; the Invasive Index; or the Migration Index are different in the presence versus the absence of a chemical.

109. The method of claim 1, wherein ~~the a first embryo incubation is in the presence or the absence of a chemical~~ with a chemical and a second embryo incubation is without the chemical, and the first embryo incubation is compared to the second embryo incubation.

110. The method of claim 109, further comprising measuring an effect of the chemical on the circulating tumor cells by:

- a. Digesting the embryo and the circulating tumor cells in a protease solution after incubation;
- b. Dispersing the embryo and the circulating tumor cells with pipetting to dissociate to a single cell suspension;

- c. Fixing and counting the circulating tumor cells in the single cell suspension under a fluorescent microscope;
 - d. Calculating a ratio of the counted circulating tumor cells to the injected circulating 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 circulating tumor cells.
- ~~12~~11. The method of claim ~~10~~9, further comprising measuring and comparing the patterns of invasiveness of circulating tumor cells in the presence or absence of the chemical.
- ~~13~~12. The method of claim 1, further comprising assessing changes in the circulating tumor cells' DNA by:
- a. enzymatically digesting the embryo and the circulating tumor cells after incubation;
 - b. isolating the digested embryo and circulating tumor cells DNA;
 - c. PCR amplifying one or more genes from the digested embryo and circulating tumor cells DNA;
 - d. Sequencing the amplified one or more genes; and
 - e. Bisulfite sequencing the digested embryo and circulating tumor cells DNA to locate epigenetic modifications.
- ~~14~~13. The method of claim 1, further comprising analyzing circulating tumor cells' gene expression by:
- a. Enzymatically digesting the embryo and the circulating tumor cells after incubation;
 - b. Isolating the digested embryo and the circulating 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~~14. The method of claim 1, further comprising analyzing circulating 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 circulating tumor cells' protein expression;
 - b. Visualizing circulating tumor cells' protein expression using immunohistochemistry on histological section slides of the embryo after circulating tumor cell injection and incubation;
 - c. Visualizing circulating tumor cells' protein expression using ELISA (enzyme-linked immunosorption assay); and
 - d. Visualizing circulating tumor cells' protein expression using Western blot.
- ~~16~~15. 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 circulating tumor cells.
- ~~17~~16. The method of claim ~~16~~15, wherein the pro-angiogenic factor is angiopoietin.
- ~~18~~17. The method of claim 1, further comprising:
- a. the cell tracking dye has a red fluorescence;
 - b. the circulating 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 circulating 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 circulating tumor cells' foci position in the image;
 - e. using the analysis of the fluorescent images to calculate a Homing Index of the circulating tumor cells as: Homing Index (HI) = $1/n \sum (\text{Total number of } \underline{\text{circulating}} \text{ tumor cells foci in an organ at T hours} / \text{Total number of } \underline{\text{circulating}} \text{ tumor cells foci at time T hours})$, where n is the number of embryos considered in the experiment, and T is the incubation time T.

~~19~~18. The method of claim ~~18~~17, further comprising observing the organ-homing pattern change of the injected circulating 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~~19. A multi-well microinjection system ~~automating microinjection of 24 to 72 hpf zebrafish embryos to inject the circulating tumor cells as claimed in the methods of claims 1 to 18~~ 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~~20. The multi-well microinjection system of claim ~~21~~19, 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~~21. The multi-well microinjection system of claim ~~21~~19, wherein the automation of the microinjection is performed using a micropipette which is controlled by a robotic arm.

~~24~~22. The multi-well microinjection system of claim ~~21~~19, 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~~23. The multi-well microinjection system of claim ~~21~~19, wherein the position and/or angle of the robotic arm is adjustable, either manually or according to a software control interface.

~~26~~24. A method for testing a drug for its effect on circulating tumor cells, comprising:

- a. microinjecting one or more circulating tumor cells into a 24 to 72 hour post-fertilization zebrafish embryo;
- b. allowing the circulating 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 circulating tumor cells by measuring the circulating 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~~25. The method of claim ~~26~~24, 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 circulating tumor cells.

~~29~~26. The method of claim ~~28~~25, wherein the pro-angiogenic factor is angiopoietin.

~~30~~27. The method of claim ~~28~~24, further comprising the addition of a pro-angiogenic factor by microinjection into the embryo during the microinjection of the circulating tumor cells; wherein the pro-angiogenic factor is microinjected into the embryo.