The present invention also provides methods for identifying agents that modulate cardiac contractility.

Title: ZEBRAFISH MODELS FOR ANALYSIS OF HEART FUNCTION
ZEBRAFISH MODELS FOR ANALYSIS OF HEART FUNCTION

This application claims the benefit of U.S. Provisional Application No. 60/814,464 filed June 16, 2006 and U.S. Provisional Application No. 60/836,515, filed August 9, 2006, both of which are incorporated herein by reference in their entireties.

BACKGROUND OF THE INVENTION

A number of pharmaceuticals have been withdrawn from the market due to cardiac toxicity. The most common and most serious cause for drug withdrawal due to cardiac effects is Torsade de Pointes (TdP), a potentially life-threatening cardiac arrhythmia. To date, all reported cases of TdP have been linked to blockage of the KCNH2 potassium channel. KCNH2, commonly known as hERG (human ether-a-go-go related gene), is one of the potassium channels that conduct the outward current responsible for repolarizing the cardiomyocyte membrane potential. Blockage of this channel can result in a prolonged interval between ventricular depolarization and ventricular repolarization (QT interval on an electrocardiogram), bradycardia, and in serious cases, arrhythmia.

Withdrawal of a drug from the market can be costly in terms of sales, reputation, and legal liability for the drug manufacturer. For this reason, drug developers are now screening compounds early in preclinical development for cardiac toxicity. Current screening strategies include in vitro electrophysiology experiments to detect drug effects on ion channel function, especially KCNH2, ex vivo experiments involving isolated perfused hearts and isolated purkinje fibers, and in vivo experiments on rodents and dogs. However, the in vitro experiments suffer from a lack of biological complexity and the ex vivo and in vivo experiments are low-throughput. Additionally, current in vivo experiments are costly and require large quantities of test compound.

The zebrafish has emerged as an excellent model for studying cardiac function and drug-induced cardiac irregularities, including toxicity. Advantages of this system include ease of genetic modification, rapid and easily observable development, and low amount of drug required. Furthermore, heart development, function and response to cardiotoxic drugs are conserved between humans and zebrafish. For instance, it has been shown that when zebrafish embryos were screened for the effects of 100...
small molecules on heart rate, 22 of 23 compounds that cause repolarization abnormalities in humans caused bradycardia in zebrafish. Similarly, it has been shown that QT-prolonging drugs, when tested in zebrafish, induced both bradycardia and arrhythmia, ranging from 2:1 AV block to more serious irregular heartbeats.

Additionally, an automated assay to detect drugs that effect heart rate was developed using transgenic heart-specific fluorescent zebrafish. However, this assay only analyzed heart rates, and many drugs reduce heart rate, without affecting the rhythmicity of the heart beat. The present invention overcomes this difficulty by providing methods that do not rely solely on heart rate analysis, but instead analyze heart beat patterns to detect irregularities.

In addition to determining the effect of a drug on heart rate pattern, determining whether a drug will affect cardiac contractility, the forcefulness with which the heart beats, is a critical part of drug-safety analysis. There are several drugs on the market whose intended pharmacological action includes changing the inotropic state of the heart (contractility). If these drugs are used in combination with a drug that has unrecognized effects on contractility, serious complications, including death, can result. Some negative inotropic drugs, including calcium channel blockers can cause bradyarrhythmia, while others, such as β adrenergic receptor antagonists, can precipitate heart failure in patients with impaired left ventricular systolic function. Positive inotropic drugs have also been associated with arrhythmia.

As stated above, zebrafish have emerged as a valuable model for studying disease and toxicity (reviewed by Doan et al., 2004 and Rubinstein, 2003 and 2006). Zebrafish are also amenable to high-throughput compound screening (Peterson et al., 2000; Burns et al., 2005). The zebrafish is therefore an excellent candidate for developing a high-throughput in vivo screening platform for detecting drug activity and drug toxicity.

Currently available in vitro cardiac contractility models suffer from a lack of biological complexity, and existing ex vivo and in vivo models for assessing contractility are low-throughput and costly. Therefore, there is a need for an in vivo model that can assess cardiac contractility in an in vivo, high-throughput fashion. The present invention fulfills this need by providing an in vivo zebrafish model to quantify changes in cardiac contractility.
SUMMARY OF THE INVENTION

Provided herein are methods and systems for detecting arrhythmia in zebrafish. Also provided are methods and systems for classifying a heart rate pattern as arrhythmic. Further provided are methods and systems for identifying agents that cause arrhythmia. The present invention provides a method of detecting arrhythmia in a zebrafish comprising obtaining a heart rate pattern having at least one peak and one trough from a zebrafish and classifying the heart rate pattern as arrhythmic.

The present invention provides a method of classifying a heart rate pattern as arrhythmic comprising: obtaining a heart rate pattern having at least one peak and at least one trough from a zebrafish; determining at least one of the following: i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations; ii) if a magnitude of change in optical density of a region of interest within the heart (peak or trough) is a first threshold greater or smaller than an average change in optical density for the pattern; iii) if a time between peaks (t; relaxation phase) or a time between troughs (f; contraction phase) is at least a second threshold different than an average time between troughs for the pattern; and classifying the heart rate pattern as arrhythmic if any one of the determinations of is true.

The present invention also provides a method for identifying an agent that causes arrhythmia comprising: contacting a zebrafish with a test agent; obtaining a heart rate pattern having at least one peak and at least one trough from the zebrafish contacted with the test agent; determining at least one of the following: i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations; ii) if a magnitude of change in optical density of a region of interest within the heart (peak or trough) is a first threshold greater or smaller than an average change in optical density for the pattern; iii) if a time between peaks (t; relaxation phase) or a time between troughs (f; contraction phase) is at least a second threshold different than an average time between troughs for the pattern; and; classifying the heart rate pattern as arrhythmic if any one of the determinations of is true, such that if the heart rate pattern is classified as arrhythmic, the test agent is an agent that causes arrhythmia.

Further provided is a method of classifying a heart rate pattern as arrhythmic comprising: obtaining a heart rate pattern having at least one peak and at least one trough from a zebrafish; determining at least one of the following: i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations; ii) if a magnitude of change in a morphological characteristic (peak or trough) is a first threshold greater
or smaller than an average change in a morphological characteristic for the pattern; iii) if a time between peaks (t; relaxation phase) or a time between troughs (f; contraction phase) is at least a second threshold different than an average time between troughs for the pattern; and classifying the heart rate pattern as arrhythmic if any one of the determinations is true.

The methods and systems can classify heart patterns as arrhythmic by receiving a heart rate pattern having at least one peak and at least one trough, determining at least one of the following: i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations, ii) if a magnitude of change in a fluorescence intensity of a heartbeat (peak or trough) is a first threshold greater or smaller than an average heartbeat for the pattern or iii) if a time between peaks (t; relaxation phase) or a time between troughs (f; contraction phase) is at least a second threshold different (longer or shorter) than an average time between troughs for the pattern, and classifying the heart rate pattern as arrhythmic if any of the determinations is true.

Further provided by this invention is a method of classifying a heart rate pattern as arrhythmic comprising: obtaining a heart rate pattern having at least one peak and at least one trough from a transgenic zebrafish that expresses a fluorescent reporter protein in the heart, determining at least one of the following: i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations, ii) if a magnitude of change in a fluorescence intensity of a heartbeat (peak or trough) is a first threshold greater or smaller than an average heartbeat for the pattern or iii) if a time between peaks (t; relaxation phase) or a time between troughs (V; contraction phase) is at least a second threshold different than an average time between troughs for the pattern; and classifying the heart rate pattern as arrhythmic if any of the determinations is true.

Also provided by the present invention is a method for identifying an agent that causes arrhythmia comprising: contacting a transgenic zebrafish that expresses a fluorescent reporter protein in the heart with a test agent; obtaining a heart rate pattern having at least one peak and at least one trough from the zebrafish contacted with the test compound, determining at least one of the following: i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations, ii) if a magnitude of change in a fluorescence intensity of a heartbeat (peak or trough) is a first threshold greater or smaller than an average heartbeat for the pattern, iii) if a time between
peaks (t; relaxation phase) or a time between troughs (f; contraction phase) is at least a second threshold different than an average time between troughs for the pattern; and classifying the heart rate pattern as arrhythmic if any of the determinations is true, such that if the heart pattern is classified as arrhythmic, the test agent is an agent that causes arrhythmia.

The methods and systems described herein can also classify a heart rate pattern as arrhythmic by receiving a heart rate pattern, determining a feature vector for the heart rate pattern, and classifying the heart rate pattern as arrhythmic based on the feature vector using a trained classifier.

The present invention also provides a method of identifying an agent that modulates cardiac contractility comprising: a) contacting a zebrafish with a test agent; b) measuring cardiac contractility; and c) determining the effect of the test agent on cardiac contractility such that if there is a difference in cardiac contractility between the zebrafish contacted with the test agent and a zebrafish not contacted with the test agent, the test agent is an agent that modulates cardiac contractility.

Additional advantages of the invention will be set forth in part in the description which follows or may be learned by practice of the invention. The advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

**BRIEF DESCRIPTION OF THE DRAWINGS**

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate embodiments of the invention and together with the description, serve to explain the principles of the invention.

Figure 1 is an exemplary operating environment.

Figure 2 is a method for classifying a heart pattern as arrhythmic.

Figure 3 is a method of classifying a heart pattern as arrhythmic based on a feature vector using a trained classifier.

Figure 4 is a heart rate pattern from a zebrafish treated with 100 µM
desipramine. The peak, trough, relaxation phase (τ), and contraction phase (τ') are shown.

Figures 5-6 are heart rate patterns from zebrafish treated with astemizole.
Figures 7-12 are heart rate patterns from zebrafish treated with bepridil.
Figures 13-17 are heart rate patterns from zebrafish treated with cisapride.
Figures 18-20 are heart rate patterns from zebrafish treated with desipramine.
Figures 21-24 are heart rate patterns from zebrafish treated with fluoxetine.
Figures 25-28 are heart rate patterns from zebrafish treated with haloperidol.
Figures 29-33 are heart rate patterns from zebrafish treated with ketoconazole.
Figures 34-36 are heart rate patterns from a zebrafish treated with loratadine.
Figures 37-38 are heart rate patterns from a zebrafish treated with pimozide.
Figures 39-41 are heart rate patterns from a zebrafish treated with propafenone.
Figures 42-49 are heart rate patterns from a zebrafish treated with terfenadine.

Figure 50 is an exemplary operating environment for the cardiac contractility 
assays described herein.

Figure 51 provides an example of measurement of cardiac contractility in 
zebrafish. Zebrafish were treated at 2dpf with either 100 µM isoproterenol or 10 µM 
verapamil. At 3dpf, contractility was assessed by measuring ventricular shortening 
fraction (VSF). The lines were drawn and measured to determine ventricular length at 
diastole and systole (left panel). Isoproterenol, a positive inotropic drug, significantly 
increased VSF, while verapamil, a negative inotropic drug, significantly decreased 
VSF compared to negative controls (right panel). Data are expressed as mean +/- 
SEM. n=3 for each condition and * represents p<0.05 compared to Control by 
student's t test.

DETAILED DESCRIPTION OF THE INVENTION

Before the present methods and systems are disclosed and described, it is to be 
understood that this invention is not limited to specific methods, specific components, 
or to particular compositions, as such may, of course, vary. It is also to be understood 
that the terminology used herein is for the purpose of describing particular 
embodiments only and is not intended to be limiting.
As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another
embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other
endpoint.

"Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

The present invention may be understood more readily by reference to the following detailed description of preferred embodiments of the invention and the Examples included therein and to the Figures and their previous and following description.

The present invention provides a method of detecting arrhythmia in a zebrafish comprising obtaining a heart rate pattern having at least one peak and one trough from a zebrafish and classifying the heart rate pattern as arrhythmic.

As utilized herein, "arrhythmic" or "arrhythmia" is defined as an abnormal heart rhythm or an irregular heartbeat pattern. Therefore, the methods of the present invention can be used to detect an arrhythmia associated with an irregular heartbeat pattern. The methods utilized herein can be utilized to detect any type of arrhythmia, including, but not limited to torsade de pointe, ventricular fibrillation, ventricular tachycardia, atrial fibrillation/flutter, multifocal atrial tachycardia, paroxysmal supraventricular tachycardia, 2:1 AV block, 3:1 AV block, Wolff-Parkinson-White syndrome, tachycardia, bradycardia, sick sinus syndrome and ectopic heartbeat.

The methods of the present invention can also be utilized to detect an arrhythmic or an irregular heartbeat pattern that is not bradycardia, tachycardia, 2:1 AV block, or 3:1 AV block.

The methods of the present invention can be utilized to analyze heartbeat patterns in any zebrafish. The zebrafish utilized in the methods of the present
invention can be a wild-type zebrafish, a mutated zebrafish (for example, but not limited to, a zebrafish with a chemical mutation, a zebrafish with a radiation induced mutation or a zebrafish with a genetically engineered mutation) or a transgenic zebrafish. Therefore, heart rate patterns measurements obtained from any zebrafish can be utilized in the methods of the present invention to identify agents that modulate or affect heart rate patterns. The heart rate patterns obtained from any zebrafish can also be classified as arrhythmic or rhythmic.

If a transgenic zebrafish is utilized, this can be a transgenic zebrafish that expresses a fluorescent protein in the heart. The transgenic zebrafish can be a transient or a stable transgenic zebrafish. Expression of the fluorescent protein can be tissue specific, i.e. heart specific (see U.S. Patent No. 6,380,458 which is incorporated herein in its entirety by this reference for the purposes of describing tissue specific expression of a reporter protein in zebrafish). For example, transgenic animals that express a reporter protein, at specific sites such as the heart can be produced by introducing a nucleic acid encoding the fluorescent protein into fertilized eggs, embryonic stem cells or the germline of the animal, wherein the nucleic acid is operably linked to and under the control of a specific promoter which allows expression of the nucleic acid in specific types of cells (e.g., a promoter which allows expression primarily in the heart). The expression sequences can also include one or more of an enhancer, a silencer, necessary information processing sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites and transcriptional terminator sequences. As used herein, a protein or gene is expressed predominantly in a given tissue, cell type, cell lineage or cell, when 90% or greater of the observed expression occurs in the given tissue cell type, cell lineage or cell.

This invention also contemplates the use of a transgenic zebrafish that expresses a fluorescent protein that is under the control of heart specific expression sequences such as the zebrafish cardiac myosin light chain-2 promoter (cMLC-2) and is expressed in the heart (see Huang et al. "Conditional Expression of a Myocardium-Specific Transgene in Zebrafish Transgenic Lines" Developmental Dynamics 233:1294-1303, (2005) which is set forth herein for the use of a zebrafish cardiac myosin light chain-2 promoter that drives heart specific expression. Huang et al. is hereby incorporated in its entirety by this reference.) Another example of an expression sequence that can be utilized to drive heart specific expression is set forth herein as SEQ ID NO: 1.
A zebrafish cardiac myosin heavy chain promoter or a cardiac troponin promoter can also be utilized to drive heart specific expression of a fluorescent reporter protein.

Fluorescent reporter proteins that can be utilized in the methods of the present invention include, but are not limited to, green fluorescent protein (GFP), cyan fluorescent protein (CFP), red fluorescent protein (RFP), yellow fluorescent protein (YFP). Other examples include the green fluorescent protein from *Aequorea coerelescens* (AcGFP), green reef coral fluorescent protein (GRCFP). DsRedExpress, and other coral fluorescent proteins (for example, AmCyan, ZsGreen, ZsYellow, AsRed2, DsRed2, and HcRedl). Fluorescent proteins can be isolated from many different species, including but not limited to, *Aequorea victoria* (Chalfie, et al., 1994), *Zoanthus* species (Matz, et al., 1999), and *Renilii reniformis* (Ward and Cormier, 1979).

Also provided by the present invention is a method of classifying a heart rate pattern as arrhythmic comprising: obtaining a heart rate pattern having at least one peak and at least one trough from a zebrafish, determining at least one of the following: i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations, ii) if a magnitude of a change in optical density of a region of interest within the heart (peak or trough) is a first threshold greater or smaller than an average change in optical density for the pattern, iii) if a time between peaks (t; relaxation phase) or a time between troughs (f; contraction phase) is at least a second threshold different than an average time between troughs for the pattern, and classifying the heart rate pattern as arrhythmic if any one of the determinations is true. Optical density can be measured by utilizing methods known to those of skill in the art, for example, by utilizing the methods set forth by Milan et al., 2003. This reference is incorporated herein in its entirety as an example of how to measure optical density of a region of interest within the heart.

Further provided by the present invention is a method of classifying a heart rate pattern as arrhythmic comprising: obtaining a heart rate pattern having at least
one peak and at least one trough from a zebrafish, determining at least one of the following: i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations, ii) if a magnitude of a change in a morphological characteristic (peak or trough) is a first threshold greater or smaller than an average change in a morphological characteristic for the pattern, iii) if a time between peaks (t; relaxation phase) or a time between troughs (f; contraction phase) is at least a second threshold different than an average time between troughs for the pattern, and classifying the heart rate pattern as arrhythmic if any one of the determinations is true. The morphological characteristic can be, but is not limited to a change in the length of the heart, a change in the size of the perimeter of the heart or a change in projected volume. One of skill in the art would know how to measure such morphological characteristics.

Also provided herein is a method of classifying a heart rate pattern as arrhythmic comprising: obtaining a heart rate pattern having at least one peak and at least one trough from a transgenic zebrafish that expresses a fluorescent reporter protein in the heart, determining at least one of the following: i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations, ii) if a magnitude of change in a fluorescence intensity of a heartbeat (peak or trough) is a first threshold greater or smaller than an average heartbeat rate for the pattern, iii) if a time between peaks (t; relaxation phase) or a time between troughs (V; contraction phase) is at least a second threshold different than an average time between troughs for the pattern; and classifying the heart rate pattern as arrhythmic if any of the determinations is true. If none of the determinations are true, the heart rate pattern can be classified as rhythmic.

The heart pattern obtained from a zebrafish described herein can be obtained by the same person or entity classifying the heart pattern. The heart pattern can also be obtained by or received by a person, institution, computer, corporation or other entity and classified by a different person, institution, computer, corporation or other entity. The heart pattern data can be obtained from a zebrafish via a heart pattern data receiving unit. The heart pattern data receiving unit can be a scanner, a recording device (including but not limited to, a video camera, a still camera, and the like), or any input device. Once the heart pattern data is received, these data can be plotted, for example, in the case of a transgenic zebrafish expressing a fluorescent protein in the heart, as fluorescence intensity over time. By analyzing the heart data pattern, one
of skill in the art can classify the pattern as arrhythmic by determining at least one of
the following: i) if the heart rate pattern has a non-repeating pattern of contractions
and relaxations, ii) if a magnitude of change in a fluorescence intensity of a heartbeat
(peak or trough) is a first threshold greater or smaller than an average heartbeat for the
pattern, or iii) if a time between peaks (t; relaxation phase) or a time between troughs
(f; contraction phase) is at least a second threshold different (longer or shorter) than
an average time between troughs for the pattern, and classifying the heart rate pattern
as arrhythmic if any of the determinations is true. The classification of the heart
pattern can be done visually by one of skill in the art or via a heart pattern data
classifying unit.

Therefore; also provided herein is a system for classifying a heart pattern as
arrhythmic comprising a heart pattern data receiving unit and a heart pattern data
classifying unit. The heart pattern data classifying unit can determine if a heart rate
pattern has a non-repeating pattern of contractions and relaxations, determine if a
magnitude of change in a fluorescence intensity of a heartbeat (peak or trough) is a
first threshold greater or smaller than an average heartbeat for the pattern, or
determine if a time between peaks (t; relaxation phase) or a time between troughs (f;
contraction phase) is at least a second threshold different (longer or shorter) than an
average time between troughs for the pattern, and classify the heart rate pattern as
arrhythmic if any of the determinations is true. The first threshold can be at least
about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50% or any percentage in
between. The second threshold can be at least about 5%, 10%, 15%, 20%, 25%, 30%,
35%, 40%, 45%, 50% or any percentage in between. The heart rate pattern
classifying unit can further determine a magnitude of change in a fluorescence
intensity of a heartbeat. The heart rate pattern classifying unit can further determine
an average heartbeat for the pattern. The heart rate pattern classifying unit can further
determine a time between peaks. The heart rate pattern classifying unit can further
determine a time between troughs.

In a further aspect, the heart rate pattern classifying unit can further determine
a feature vector for a heart rate pattern, and classify the heart rate pattern as
arrhythmic based on the feature vector using a trained classifier. The heart rate pattern
classifying unit can further be trained by presenting, to the classifier, a pre-classified
heart rate pattern and determining, by the classifier, an optimal vector for the pre-
classified heart rate pattern from a pre-determined set of features. The classifier can
be any classifier known in the art including, but not limited to, a k-Nearest Neighbors
classifier, a linear discriminant classifier, a quadratic discriminant classifier, and a
support vector machine. The features used to train the classifier can include the heart
rate pattern having a non-repeating pattern of contractions and relaxations, a

magnitude of the change in fluorescence intensity of any heartbeat (peak or trough)
that is at least a first threshold different (greater or smaller) than the average heartbeat
for that pattern, and a time between peaks (t; relaxation phase) or a time between
troughs (f; contraction phase) that is at least a second threshold different (longer or
shorter) than the average for that pattern. The first threshold can be at least about 5%,
10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50% or any percentage in between. The
second threshold can be at least about 5%, 10%, 25%, 20%, 15%, 30%, 35%, 40%,
45%, 50% or any percentage in between.

A system has been described above as comprised of units. One skilled in the
art will appreciate that this is a functional description and that the respective functions
can be performed by software, hardware, or a combination of software and hardware.
A unit can be software, hardware, or a combination of software and hardware. The
units can comprise the Arrhythmia Classification Software 106 as illustrated in FIG.
1 and described below. In one exemplary aspect, the units can comprise a computer
101 as illustrated in FIG. 1 and described below.

FIG. 1 is a block diagram illustrating an exemplary operating environment for
performing the disclosed methods with transgenic zebrafish expressing a fluorescent
protein in the heart. One of skill in the art will understand that such an environment
can be modified to perform the methods disclosed herein with any zebrafish.
Therefore, the illustrated operating environment can be utilized to perform the
disclosed methods with nontransgenic fish in which a change in optical density of a
region of the heart or a change in a morphological characteristic of the heart is
measured. This exemplary operating environment is only an example of an operating
environment and is not intended to suggest any limitation as to the scope of use or
functionality of operating environment architecture. Neither should the operating
environment be interpreted as having any dependency or requirement relating to any
one or combination of components illustrated in the exemplary operating
environment.

The system and methods of the present invention can be operational with
numerous other general purpose or special purpose computing system environments
or configurations. Examples of well known computing systems, environments, and/or configurations that can be suitable for use with the system and method comprise, but are not limited to, personal computers, server computers, laptop devices, and multiprocessor systems. Additional examples comprise set top boxes, programmable consumer electronics (including but not limited to, Personal Digital Assistants, cellular phones, and the like), network PCs, minicomputers, mainframe computers, distributed computing environments that comprise any of the above systems or devices, and the like.

In another aspect, the system and method of the present invention can be described in the general context of computer instructions, such as program modules, being executed by a computer. Generally, program modules comprise routines, programs, objects, components, data structures, etc. that perform particular tasks or implement particular abstract data types. The system and method of the present invention can also be practiced in distributed computing environments where tasks are performed by remote processing devices that are linked through a communications network. In a distributed computing environment, program modules can be located in both local and remote computer storage media including memory storage devices.

Further, one skilled in the art will appreciate that the system and method disclosed herein can be implemented via a general-purpose computing device in the form of a computer 101. The components of the computer 101 can comprise, but are not limited to, one or more processors or processing units 103, a system memory 112, and a system bus 113 that couples various system components including the processor 103 to the system memory 112.

The system bus 113 represents one or more of several possible types of bus structures, including a memory bus or memory controller, a peripheral bus, an accelerated graphics port, and a processor or local bus using any of a variety of bus architectures. By way of example, such architectures can comprise an Industry Standard Architecture (ISA) bus, a Micro Channel Architecture (MCA) bus, an Enhanced ISA (EISA) bus, a Video Electronics Standards Association (VESA) local bus, an Accelerated Graphics Port (AGP) bus, and a Peripheral Component Interconnects (PCI) bus also known as a Mezzanine bus. The bus 113, and all buses specified in this description can also be implemented over a wired or wireless network connection and each of the subsystems, including the processor 103, a mass storage device 104, an operating system 105, Arrhythmia Prediction software 106, heart
pattern data 107, a network adapter 108, system memory 112, an Input/Output Interface 110, a display adapter 109, a display device 111, and a human machine interface 102, can be contained within one or more remote computing devices 114a,b,c at physically separate locations, connected through buses of this form, in effect implementing a fully distributed system.

The computer 101 typically comprises a variety of computer readable media. Exemplary readable media can be any available media that is accessible by the computer 101 and comprises, for example and not meant to be limiting, both volatile and non-volatile media, removable and non-removable media. The system memory 112 comprises computer readable media in the form of volatile memory, such as random access memory (RAM), and/or non-volatile memory, such as read only memory (ROM). The system memory 112 typically contains data such as heart pattern data 107 and/or program modules such as operating system 105 and Arrhythmia Classification software 106 that are immediately accessible to and/or are presently operated on by the processing unit 103.

In another aspect, the computer 101 can also comprise other removable/non-removable, volatile/non-volatile computer storage media. By way of example, FIG. 1 illustrates a mass storage device 104 which can provide non-volatile storage of computer code, computer readable instructions, data structures, program modules, and other data for the computer 101. For example and not meant to be limiting, a mass storage device 104 can be a hard disk, a removable magnetic disk, a removable optical disk, magnetic cassettes or other magnetic storage devices, flash memory cards, CD-ROM, digital versatile disks (DVD) or other optical storage, random access memories (RAM), read only memories (ROM), electrically erasable programmable read-only memory (EEPROM), and the like.

Optionally, any number of program modules can be stored on the mass storage device 104, including by way of example, an operating system 105 and Arrhythmia Classification software 106. Each of the operating system 105 and Arrhythmia Classification software 106 (or some combination thereof) can comprise elements of the programming and the Arrhythmia Classification software 106. Heart pattern data 107 can also be stored on the mass storage device 104. Heart pattern data 107 can be stored in any of one or more databases known in the art. Examples of such databases comprise, DB2®, Microsoft® Access, Microsoft® SQL Server, Oracle®, mySQL,
PostgreSQL, and the like. The databases can be centralized or distributed across multiple systems.

In another aspect, the user can enter commands and information into the computer 101 via an input device (not shown). Examples of such input devices comprise, but are not limited to, a keyboard, pointing device (e.g., a "mouse"), a microphone, a joystick, a scanner, and the like. These and other input devices can be connected to the processing unit 103 via a human machine interface 102 that is coupled to the system bus 113, but can be connected by other interface and bus structures, such as a parallel port, game port, an IEEE 1394 Port (also known as a Firewire port), a serial port, or a universal serial bus (USB).

In yet another aspect of the present invention, a display device 111 can also be connected to the system bus 113 via an interface, such as a display adapter 109. It is contemplated that the computer 101 can have more than one display adapter 109 and the computer 101 can have more than one display device 111. For example, a display device can be a monitor, an LCD (Liquid Crystal Display), or a projector. In addition to the display device 111, other output peripheral devices can comprise components such as speakers (not shown) and a printer (not shown) which can be connected to the computer 101 via Input/Output Interface 110.

The computer 101 can operate in a networked environment using logical connections to one or more remote computing devices 114a,b,c. By way of example, a remote computing device can be a personal computer, portable computer, a server, a router, a network computer, a peer device or other common network node, and so on. Logical connections between the computer 101 and a remote computing device 114 a,b,c can be made via a local area network (LAN) and a general wide area network (WAN). Such network connections can be through a network adapter 108. A network adapter 108 can be implemented in both wired and wireless environments. Such networking environments are conventional and commonplace in offices, enterprise-wide computer networks, intranets, and the Internet 115.

For purposes of illustration, application programs and other executable program components such as the operating system 105 are illustrated herein as discrete blocks, although it is recognized that such programs and components reside at various times in different storage components of the computing device 101, and are executed by the data processors) of the computer. An implementation of Arrhythmia Classification software 106 can be stored on or transmitted across some form of
computer readable media. Computer readable media can be any available media that can be accessed by a computer. By way of example and not meant to be limiting, computer readable media can comprise "computer storage media" and "communications media." "Computer storage media" comprise volatile and non-volatile, removable and non-removable media implemented in any method or technology for storage of information such as computer readable instructions, data structures, program modules, or other data. Exemplary computer storage media comprises, but is not limited to, RAM, ROM, EEPROM, flash memory or other memory technology, CD-ROM, digital versatile disks (DVD) or other optical storage, magnetic cassettes, magnetic tape, magnetic disk storage or other magnetic storage devices, or any other medium which can be used to store the desired information and which can be accessed by a computer.

The methods and systems of the present invention can employ Artificial Intelligence techniques such as machine learning and iterative learning. Examples of such techniques include, but are not limited to, expert systems, case based reasoning, Bayesian networks, behavior based AI, neural networks, fuzzy systems, evolutionary computation (e.g. genetic algorithms), swarm intelligence (e.g. ant algorithms), and hybrid intelligent systems (e.g. Expert inference rules generated through a neural network or production rules from statistical learning). Supervised learning is a machine learning technique for creating a function from training data. The training data can comprise input objects (typically vectors), and desired outputs. The input object can comprise either normal (rhythmic) or arrhythmic heart rate patterns. The output of the function can be a continuous value (regression), or can predict a class label of the input object (classification). The output can be a determination that a heart rate pattern is normal (rhythmic), or that a heart rate pattern is arrhythmic. The task of the supervised learner is to predict the value of the function for any valid input object after having seen a number of training examples (i.e. pairs of input and target output). To achieve this, the learner has to generalize from the presented data to unseen situations in a "reasonable" way (inductive bias). Supervised learning can generate models of two types. Most commonly, supervised learning generates a global model that maps input objects to desired outputs. In some cases, however, the map is implemented as a set of local models (such as in case-based reasoning or the nearest neighbor algorithm).
The processing of the disclosed system and methods of the present invention can be performed by software components. The disclosed system and methods can be described in the general context of computer-executable instructions, such as program modules, being executed by one or more computers or other devices. Generally, program modules comprise computer code, routines, programs, objects, components, data structures, etc. that perform particular tasks or implement particular abstract data types. The disclosed methods can also be practiced in grid-based and distributed computing environments where tasks are performed by remote processing devices that are linked through a communications network. In a distributed computing environment, program modules can be located in both local and remote computer storage media including memory storage devices.

In one aspect, as shown in FIG. 2, provided is a method for classifying a heart pattern as arrhythmic comprising receiving a heart rate pattern having at least one peak and at least one trough at block 201, determining at least one of the following in blocks 202, 203 or 204 (determining if the heart rate pattern has a non-repeating pattern of contractions and relaxations at block 202, determining if a magnitude of change in a fluorescence intensity of a heartbeat (peak or trough) is a first threshold greater or smaller than an average heartbeat for the pattern at block 203, determining if a time between peaks (t; relaxation phase) or a time between troughs (f; contraction phase) is at least a second threshold different (longer or shorter) than an average time between troughs for the pattern at block 204), and classifying the heart rate pattern as arrhythmic if any of the determinations is true at block 205. The first threshold can be at least about 5%, 10%, 15%, 20%, 25% 30%, 35%, 40%, 45%, 50% or any percentage in between. The second threshold can be at least about 5%, 10%, 15%, 20%, 25% 30%, 35%, 40%, 45%, 50% or any percentage in between. The method can further comprise determining a magnitude of change in a fluorescence intensity of a heartbeat. The method can further comprise determining an average heartbeat for the pattern. The method can further comprise determining a time between peaks. The method can further comprise determining a time between troughs.

In a further aspect, as shown in FIG. 3, provided is an automated method for classifying a heart rate pattern as arrhythmic comprising receiving a heart rate pattern at block 301, determining a feature vector for the heart rate pattern at block 302, and classifying the heart rate pattern as arrhythmic based on the feature vector using a trained classifier at block 303. Training the classifier can further comprise presenting,
to the classifier, a pre-classified heart rate pattern and determining, by the classifier, an optimal vector for the pre-classified heart rate pattern from a pre-determined set of features. The classifier can be any classifier known in the art including, but not limited to, a k-Nearest Neighbors classifier, a linear discriminant classifier, a quadratic discriminant classifier, and a support vector machine. The features used to train the classifier can include the heart rate pattern having a non-repeating pattern of contractions and relaxations, a magnitude of the change in fluorescence intensity of any heartbeat (peak or trough) is at least a first threshold different (greater or smaller) than the average heartbeat for that pattern, and a time between peaks (t; relaxation phase) or a time between troughs (f; contraction phase) is at least a second threshold different (longer or shorter) than the average for that pattern. The first threshold can be at least about 5%, 10%, 15%, 20%, 25% 30%, 35%, 40%, 45%, 50% or any percentage in between. The second threshold can be at least about 5%, 10%, 15%, 20%, 25% 30%, 35%, 40%, 45%, 50% or any percentage in between.

Also provided herein is a method for identifying an agent that causes arrhythmia comprising: contacting a zebrafish with a test agent; obtaining a heart rate pattern having at least one peak and at least one trough from the zebrafish contacted with the test compound; determining at least one of the following: i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations, ii) if a magnitude of a change in optical density of a region of interest within the heart (peak or trough) is a first threshold greater or smaller than an average change in optical density for the pattern, iii) if a time between peaks (t; relaxation phase) or a time between troughs (f; contraction phase) is at least a second threshold different than an average time between troughs for the pattern; and classifying the heart rate pattern as arrhythmic if any of the determinations is true, such that if the heart pattern is classified as arrhythmic, the test agent is an agent that causes arrhythmia.

Also provided herein is a method for identifying an agent that causes arrhythmia comprising: contacting a zebrafish with a test agent; obtaining a heart rate pattern having at least one peak and at least one trough from a zebrafish, determining at least one of the following: i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations, ii) if a magnitude of a change in a morphological characteristic (peak or trough) is a first threshold greater or smaller than an average change in a morphological characteristic for the pattern, iii) if a time between peaks (t; relaxation phase) or a time between troughs (f; contraction phase) is at least a
second threshold different than an average time between troughs for the pattern, and
classifying the heart rate pattern as arrhythmic if any of the determinations is true,
such that if the heart pattern is classified as arrhythmic, the test agent is an agent that
causes arrhythmia.

Also provided herein is a method for identifying an agent that causes
arrhythmia comprising: contacting a transgenic zebrafish that expresses a fluorescent
reporter protein in the heart with a test agent; obtaining a heart rate pattern having at
least one peak and at least one trough from the zebrafish contacted with the test
compound; determining one of the following: i) if the heart rate pattern has a non-
repeating pattern of contractions and relaxations, ii) if a magnitude of change in a
fluorescence intensity of a heartbeat (peak or trough) is a first threshold greater or
smaller than an average heartbeat for the pattern, iii) determining if a time between
peaks (t; relaxation phase) or a time between troughs (t\(^1\); contraction phase) is at least
a second threshold different than an average time between troughs for the pattern; and
classifying the heart rate pattern as arrhythmic if any of the determinations is true,
such that if the heart pattern is classified as arrhythmic, the test agent is an agent that
causes arrhythmia.

In all of the methods described herein for identifying agents that cause
arrhythmia, the heart rate pattern of the zebrafish contacted with the test compound
can be compared with the heart rate pattern of a zebrafish not contacted with the test
compound, i.e., a control zebrafish, in order to assess the arrhythmic effects of the
agent. A control zebrafish can also be a zebrafish that does not exhibit arrhythmia in
the absence of a test compound. It is understood that a control zebrafish is a zebrafish
that is essentially similar to the zebrafish contacted with the test agent, except that it
was not contacted with the test agent. For example, a control zebrafish for a
transgenic zebrafish expressing a fluorescent protein in the heart that is contacted with
a test agent, is a transgenic zebrafish expressing a fluorescent protein in the heart that
is not contacted with the test agent. One of skill in the art can also compare the heart
rate pattern of a zebrafish contacted with a test agent to the heart rate pattern of a
zebrafish contacted with an agent that is known to have arrhythmic effects. For
example, one of skill in the art can compare a zebrafish contacted with a test agent to
a zebrafish contacted with an arrhythmic drug. If the test agent causes arrhythmia,
one of skill in the art can then determine to what extent its effect (increasing
arrhythmia) is greater than, equal to or less than the known arrhythmic drug's effect.
Similarly, one of skill in the art can compare a zebrafish contacted with a test agent to a zebrafish contacted with a drug that reduces arrhythmia. If the test agent reduces arrhythmia, one of skill in the art can then determine to what extent its effect (decreasing arrhythmia) is greater than, equal to or less than the known drug's effect. Such comparisons can be performed with any drug, currently known or identified in the future, including those agents identified utilizing the methods of the present invention. A database of results achieved with arrhythmic or antiarrhythmic agents can be established such that one of skill the art can compare the effects of test agents with known arrhythmic and antiarrhythmic agents.

The test agents or compounds used in the methods described herein can be, but are not limited to, drugs, morpholinos, chemicals, hormones, small molecules, antibodies, cDNAs encoding proteins, antisense molecules, siRNAs, peptides and secreted proteins. The zebrafish can be soaked in the test compound or injected with the test compound. Test compounds can be injected into the yolk, introduced into the blood stream by injecting into the heart cavity, injected into the gut or injected intramuscularly or injected in any other way that introduces the agent into the zebrafish. Libraries of compounds can be tested by arraying zebrafish in multi-well plates and administering compounds in small volumes to each well. The wells utilized in the methods described herein can hold about 10 µl 25 µl, 50 µl, 100 µl, 200 µl, 300 µl, 500 µl, 750 µl, 1 ml, 2 ml, 3 ml, 5ml, any amount of media in between these amounts or greater than 5 ml. Therefore, the methods described herein can be used to provide a high throughput assay for evaluating the effects of drugs or combinations of drugs on heart rate patterns and/or cardiac contractility.

Any of the methods described herein for assessing heart rate patterns or cardiac contractility can be performed in parallel, for example, by using an array. For example, a plurality of zebrafish in an array can be contacted with different agents, different concentrations of the same agent, or the same agent in combination with different second agents. Each position (such as a well or unpartitioned region) on an array can include one or more zebrafish.

In the methods of the present invention, the effect of a first test agent can be evaluated in combination with at least a second test agent. The first and second test agents can be contacted simultaneously with the zebrafish. The first and second agents can also be contacted consecutively, and the evaluation of the heart rate pattern and/or cardiac contractility performed before, after, and/or between contacting of the
first and second agent. In another example, the first agent may be removed before the second agent is contacted with the zebrafish.

The methods of the present invention can optionally comprise contacting the zebrafish with a substance (e.g., a dye, for example, a voltage sensitive dye) that allows more sensitive visualization of the cardiovascular system. The dye can be a calcium responsive dye.

By utilizing the methods of the present invention, one of skill in the art can determine the effect of an agent or compound on heartbeat patterns. Utilizing the methods set forth herein, Applicants have shown the effects of drugs set forth in the Examples on heartbeat patterns. Figures 5-44 provide numerous examples of rhythmic and arrhythmic patterns obtained utilizing the methods of the present invention.

The effects of an agent, if any, can be dose dependent. For example, some agents will produce an arrhythmic pattern at very low dosages. This will be indicative to one of skill in the art that the agent has a very high arrhythmic potential. Other agents may not produce an arrhythmic pattern at any dosage level. One of skill would recognize this agent as an agent with lowered or no arrhythmic potential. In another example, an agent may produce a rhythmic pattern at lower dosages and an arrhythmic pattern at higher dosages. One of skill in the art would recognize that this test agent has increased arrhythmic potential at higher dosages, but has decreased arrhythmic potential at lower dosages. Therefore, one of skill in the art can determine a dosage range for this agent that does not result in increased arrhythmic potential for a subject, by categorizing the agent as an agent that can be administered below a certain dosage level, i.e. a threshold level, for increased arrhythmic potential.

In another example, it is also possible for an agent to produce a rhythmic pattern in one zebrafish and an arrhythmic pattern in another zebrafish at the same dosage level. This is consistent with humans, where only some patients develop arrhythmia in response to drugs with negative cardiac effects. In this instance, it would be clear to one of skill in the art that the agent may have arrhythmic potential for some individuals at a certain dosage level. One of skill in the art could then increase the dosage to determine if upon increasing the dosage of the agent more zebrafish exhibit arrhythmia. One of skill in the art could also decrease the dosage to determine if upon decreasing the dosage of the agent, fewer zebrafish exhibit arrhythmias. This would allow one of skill in the art to establish a dosage range for
the agent corresponding to decreased arrhythmic potential across a population of zebrafish.

Any of the agents identified as producing an arrhythmic pattern utilizing the methods of the present invention can be administered to another model for detection of arrhythmia such as a mouse model, a rat model etc. to determine if the agent produces an arrhythmic pattern in a non-zebrafish model. Similarly, any of the agents identified as producing a rhythmic pattern can be administered to another model such as a mouse model, a rat model etc. to determine if the agent produces a rhythmic pattern in a non-zebrafish model. Ultimately, any of the threshold levels or dosage ranges established using the methods of the present invention can be utilized to establish dosage ranges for administration to any subject, for example, a mammal, preferably human, and can include, but is not limited to mouse, rat, cow, guinea pig, hamster, rabbit, cat, dog, goat, sheep, monkey, horse and chimpanzee. Furthermore, the results obtained utilizing the methods of the present invention can be used to predict whether an agent, for example, a drug, would have increased arrhythmic potential in humans. Once an agent is identified as having increased arrhythmic potential utilizing the methods set forth herein, this agent can be classified as an agent that could increase arrhythmic potential and cause arrhythmia in humans. Therefore, the methods of the present invention can also include correlating the effect of the agent on the zebrafish with a predicted effect of the agent on a mammal, for example, a human. This information identifying the effect of the agent as a possible or predicted effect of the agent in a mammal can be provided to a company, the government, a health care provider, a patient etc. Therefore, the methods set forth herein provide an efficient screening strategy that can save the pharmaceutical industry considerable time, money and effort by identifying drugs early in development that could cause cardiac abnormalities in humans.

Cardiac Contractility Assays

The present invention provides a method of identifying an agent that modulates cardiac contractility comprising: a) contacting a zebrafish with a test agent; b) measuring cardiac contractility; and determining the effect of the test agent on cardiac contractility such that if there is a difference in cardiac contractility between the zebrafish contacted with the test agent and a zebrafish not contacted with the test
agent, the test agent is an agent that modulates cardiac contractility.

As utilized herein "cardiac contractility" or "inotropic state" is a property, belonging to cardiac cells and tissues, of shortening in response to an appropriate stimulus. Cardiac contractility is variable and is influenced by the autonomic nervous system and is also affected by other factors such as loading conditions; changes in contractility give rise to changes in the strength of contraction of the heart. Thus, cardiac contractility can also be defined as the forcefulness with which the heart beats.

The methods of the present invention can be utilized to measure cardiac contractility in any zebrafish. The zebrafish utilized in the methods of the present invention can be a wild-type zebrafish, a mutated zebrafish (for example, but not limited to, a zebrafish with a chemical mutation, a zebrafish with a radiation induced mutation or a zebrafish with a genetically engineered mutation) or a transgenic zebrafish. Therefore, cardiac contractility measurements obtained from any zebrafish can be utilized in the methods of the present invention to identify agents that modulate or affect cardiac contractility.

The transgenic zebrafish utilized in the methods set forth herein can be a transgenic zebrafish that expresses a fluorescent protein in the heart. The transgenic zebrafish can be a transient or a stable transgenic. Expression of the fluorescent protein can be tissue specific, i.e. heart specific (see U.S. Patent No. 6,380,458 which is incorporated herein in its entirety by this reference for the purposes of describing tissue specific expression of a reporter protein in zebrafish). For example, transgenic animals that express a reporter protein, at specific sites such as the heart can be produced by introducing a nucleic acid encoding the fluorescent protein into fertilized eggs, embryonic stem cells or the germline of the animal, wherein the nucleic acid is operably linked to and under the control of a specific promoter which allows expression of the nucleic acid in specific types of cells (e.g., a promoter which allows expression primarily in the heart). The expression sequences can also include an enhancer, a silencer and necessary information processing sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites and transcriptional terminator sequences. As used herein, a protein or gene is expressed predominantly in a given tissue, cell type, cell lineage or cell, when 90% or greater of the observed expression occurs in the given tissue cell type, cell lineage or cell.

This invention also contemplates the use of a transgenic zebrafish that expresses a fluorescent protein that is under the control of heart specific expression
sequences such as the zebrafish cardiac myosin light chain-2 promoter (cMLC-2) and is expressed in the heart (see Huang et al. "Conditional Expression of a Myocardium-Specific Transgene in Zebrafish Transgenic Lines" Developmental Dynamics 233:1294-1303, (2005) which is set forth herein for the use of a zebrafish cardiac myosin light chain-2 promoter that drives heart specific expression. Huang et al. is hereby incorporated in its entirety by this reference.) Another example of an expression sequence that can be utilized is set forth herein as SEQ ID NO: 1.

(SEQ ID NO: 1) gtcctccctcc ccactctgcac acttttatc ctttccaccc tggctggaat
gtagcacttc gtagcattat cagggctcct gtatgttagga gccctgtgggt gttcatgtag gggacgaaca
gaaactgtc agaaccttata gaagaacaac

A zebrafish cardiac myosin heavy chain promoter, a BMP4 promoter, or a cardiac troponin promoter can also be utilized to drive heart specific expression of a fluorescent reporter protein. Promoters that drive ventricle specific expression or atrium specific expression can also be utilized.

Fluorescent reporter proteins that can be utilized in the methods of the present invention include, but are not limited to, green fluorescent protein (GFP), cyan fluorescent protein (CFP), red fluorescent protein (RFP), and yellow fluorescent protein (YFP). Other examples include the green fluorescent protein from Aequorea coerelescens (AcGFP), green reef coral fluorescent protein (GRCFP), DsRedExpress, and other coral fluorescent proteins (for example, AmCyan, ZsGreen, ZsYellow, AsRed2, DsRed2, and HcRedl). Fluorescent proteins can be isolated from many different species, including but not limited to, Aequorea victoria (Chalfie, et al., 1994), Zoanthus species (Matz, et al., 1999), and Renilla reniformis (Ward and Cormier, 1979).

The test agents or compounds used in the methods described herein can be, but are not limited to, drugs, chemicals, small molecules, antibodies, cDNAs encoding proteins, morpholinos, peptide nucleic acids and other antisense molecules, siRNAs, ribozymes, small peptides, large peptides and secreted proteins. The zebrafish can be contacted with one or more test agents. The zebrafish can be soaked in the test compound or injected with the test compound. Test compounds can be injected into the yolk, introduced into the blood stream by injecting into the heart cavity, injected into the gut or injected intramuscularly or injected in any other way that introduces the agent into the zebrafish. Libraries of compounds can be tested by arraying zebrafish in multi-well plates and administering compounds in small volumes to each...
well. Libraries can also be injected into the zebrafish.

There are numerous methods known to one of skill in the art for measuring cardiac contractility in any zebrafish, including a wild-type zebrafish, a nontransgenic zebrafish, a mutated zebrafish or a transgenic zebrafish. These include, but are not limited to, calculating or measuring ventricular shortening fraction, calculating the percent change in ventricular area during a heartbeat, calculating atrial shortening fraction, calculating the percent change in atrial area, calculating changes in intensity in a ventricle or atrial section. For example, one of skill in the art can record cardiac contractions using a video camera or video stream for a period of time, for example, about 0.5 seconds, about 1 second, about 2 seconds, about 3 seconds, about 5 seconds, about 10 seconds, about 15 seconds, about 30 seconds, about 45 seconds, about 1 minute, about 2 minutes, about 3 minutes, about 4 minutes, about 5 minutes, about 6 minutes, any period of time in between these periods of time, or longer than 6 minutes. The lengths of the ventricles in diastolic and systolic conditions can then be measured to calculate the ventricular shortening fraction (VSF) according to the following equation:

\[
\text{VSF} = \frac{\text{End diastolic ventricular length} - \text{End systolic ventricular length}}{\text{End diastolic ventricular length}}
\]

Similarly, one of skill in the art can record cardiac contractions using a video camera or video stream for a period of time, for example, about 0.5 seconds, about 1 second, about 2 seconds, about 3 seconds, about 5 seconds, about 10 seconds, about 15 seconds, about 30 seconds, about 45 seconds, about 1 minute, about 2 minutes, about 3 minutes, about 4 minutes, about 5 minutes, about 6 minutes, any time period in between these periods of time, or longer than 6 minutes. The area of the ventricles in diastolic and systolic conditions can then be measured to calculate the percent change in ventricular area according to the following equation:

\[
\text{% change in ventricular area} = \frac{\text{End diastolic ventricular area} - \text{End systolic ventricular area}}{\text{End diastolic ventricular area}} \times 100
\]

Atrial measurements can also be performed. For example, one of skill in the art can measure atrial shortening fraction (ASF) according to the following formula:
ASF = \frac{\text{End diastolic atrial length} - \text{End systolic atrial length}}{	ext{End diastolic atrial length}}

One of skill in the art can also calculate the percent change in atrial area according to the following equation:

\%
\text{change in atrial area} = \frac{\text{End diastolic atrial area} - \text{End systolic atrial area}}{\text{End diastolic atrial area}} \times 100

It is understood that the images of cardiac contractions can be obtained by the same person performing ventricular and/or atrial measurements, or the images can be obtained by another person or source that is not the same person performing ventricular and/or atrial measurements. In other words, one of skill in the art can obtain a video stream or images of cardiac contractions from another laboratory, institute, company, individual etc. and perform ventricular and/or atrial measurements. These images can be images recorded before or after contacting a zebrafish with a test agent. It is also understood that software can be utilized to identify heart tissue, such as a ventricle(s) and/or atrium in an image and perform the measurements described herein.

One of skill in the art can also utilize a transgenic zebrafish expressing a fluorescent protein in the heart to calculate ventricular shortening fraction, percent change in ventricular area during a heartbeat, atrial shortening fraction or percent change in atrial area during a heartbeat as described above. For example, image thresholding can be applied to video streams of treated or untreated zebrafish using MetaMorph software (Molecular Devices Corporation, Downington, PA, USA) such that only the ventricle is highlighted. The area of the ventricle is measured by the software, and recorded at the end of the diastole and at the end of systole. As utilized herein, "diastole" is the normal rhythmically occurring relaxation and dilatation of the heart chambers, especially the ventricles, during which they fill with blood. As utilized herein, "systole" is the rhythmic contraction of the heart, especially of the ventricles. The percent change in ventricular length or area can then be calculated utilizing the formulas set forth above. This can also be done for atrial measurements.
It is understood that the software that can be utilized to process images and perform these measurements can be any software now known or developed in the future that can image tissue, for example, heart tissue, a heart ventricle etc. a heart atrium, and perform morphological measurements.

By utilizing a transgenic zebrafish expressing a fluorescent protein in the heart in the methods of the present invention, one of skill in the art can also assess cardiac contractility by selecting a region of interest within a ventricle or atrium and measuring the change in fluorescence intensity of that region with each heartbeat. A larger change in fluorescence intensity can correspond to a larger contractility. For measuring contractility, the fluorescence intensity of a specified region of interest at the end of diastole and at the end of systole from each heartbeat can be determined via any means known in the art for measuring fluorescence, for example, but not limited to, fluorescence microscopy or from heartbeat plots based on intensity measurements. Intensity measurements are not limited to fluorescence intensity measurements as these methods can also be performed with wild-type or nontransgenic zebrafish by measuring the gray intensity change when using phase contrast brightfield microscopy. The zebrafish utilized in the methods described herein can also comprise an externally introduced dye or stain that allows one of skill in the art to visualize heart tissues and perform ventricular and/or atrial measurements.

As utilized herein, an agent that modulates cardiac contractility can be an agent that increases cardiac contractility or decreases cardiac contractility in a zebrafish as compared to a control zebrafish that was not contacted with the agent. Therefore, the difference in cardiac contractility between a zebrafish contacted with a test agent and a zebrafish not contacted with the test agent can be an increase in cardiac contractility or a decrease in cardiac contractility. It is understood that a control zebrafish is a zebrafish that is essentially similar to the zebrafish contacted with the test agent, except that it was not contacted with the test agent. For example, a control zebrafish for a transgenic zebrafish expressing a fluorescent protein in the heart that is contacted with a test agent, is a transgenic zebrafish expressing a fluorescent protein in the heart that is not contacted with the test agent. One of skill in the art can also compare the cardiac contractility of a zebrafish contacted with a test agent to the cardiac contractility of a zebrafish contacted with an agent that is known to modulate cardiac contractility. For example, one of skill in the art can compare a zebrafish contacted with a test agent to a zebrafish contacted with a positive inotropic
drug, for example, isoproterenol. If the test agent is a positive inotropic drug, one of skill in the art can then determine to what extent its effect (increasing cardiac contractility) is greater than, equal to or less than isoproterenol's effect. Similarly, one of skill in the art can compare a zebrafish contacted with a test agent to a zebrafish contacted with a negative inotropic drug, for example, verapamil. If the test agent is a negative inotropic drug, one of skill in the art can then determine to what extent its effect (decreasing cardiac contractility) is greater than, equal to or less than verapamil's effect. Isoproterenol and verapamil are merely examples of inotropic drugs. Such comparisons can be performed with any inotropic drug, currently known or identified in the future, including those agents identified utilizing the methods of the present invention. A database of results achieved with inotropic agents can be established such that one of skill the art can compare the effects of test agents with known inotropic agents.

An increase in cardiac contractility can be about a 0.01 fold increase, 0.05 fold increase, 0.25-fold increase, a 0.5-fold increase, 1-fold increase, a 2-fold increase, a 3-fold increase, a 4-fold increase, a 5-fold increase, a 6-fold increase, a 7-fold increase, an 8-fold increase, a 9-fold increase, a 10-fold increase, a 15-fold increase, a 20-fold increase, a 25-fold increase, a fold increase in between the fold increases described above, or greater than about a 25-fold increase as compared to a control zebrafish.

The increase in cardiac contractility can also be an increase of about 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, a percentage increase in between the percentage increases described above, or greater than about a 500% increase as compared to a control zebrafish. For example, if a control zebrafish exhibits a ventricular shortening fraction of 20 (10^-2) and a zebrafish contacted with a test agent exhibits a ventricular shortening fraction of 30 (10^-2), the zebrafish contacted with the test agent is an agent that increases ventricular shortening fraction by 50% and thus increases cardiac contractility by 50%. Similarly, if a control zebrafish exhibits a ventricular shortening fraction of 20 (10^-2) and a zebrafish contacted with a test agent exhibits a ventricular shortening fraction of 10 (10^-2), the zebrafish contacted with the test agent is an agent that decreases ventricular shortening fraction by 50% and thus decreases cardiac contractility by 50%. These calculations are merely exemplary and not meant to be limiting. It would be recognized by one of skill in the art that similar calculations and comparisons can be done for determining an increase or decrease in cardiac contractility by measuring the
percent change in ventricular area or measuring the change in intensity of a region within a ventricle or atrium with each heartbeat.

A decrease in cardiac contractility does not have to be complete as this decrease can range from a slight decrease in cardiac contractility to a decrease that completely eliminates cardiac contractility or the ability of a ventricle to contract. A decrease in cardiac contractility can be about a 0.1-fold decrease, 0.05-fold decrease, 0.25-fold decrease, a 0.5-fold decrease, 1-fold decrease, a 2-fold decrease, a 3-fold decrease, a 4-fold decrease, a 5-fold decrease, a 6-fold decrease, a 7-fold decrease, an 8-fold decrease, a 9-fold decrease, a 10-fold decrease, a 15-fold decrease, a 20-fold decrease, a 25-fold decrease, a fold decrease in between the fold decrease described above, or greater than about a 20-fold increase as compared to a control zebrafish. The decrease in cardiac contractility can also be a decrease of about 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, a percentage decrease in between the percentage decreases described above, or greater than about a 500% decrease as compared to a control zebrafish.

One skilled in the art will appreciate that this is a functional description and that the respective functions can be performed by software, hardware, or a combination of software and hardware. A unit can be software, hardware, or a combination of software and hardware. In other words, imaging, the contractility measurements described herein, comparison of contractility data between Zebrafish contacted with a test agent and a control zebrafish, classification of positive inotropic agents and classification of negative inotropic agents can all be performed in a computing environment comprised of software, hardware, or a combination of software and hardware. FIG. 50 is a block diagram illustrating an exemplary operating environment for performing the disclosed method. This exemplary operating environment is only an example of an operating environment and is not intended to suggest any limitation as to the scope of use or functionality of operating environment architecture. Neither should the operating environment be interpreted as having any dependency or requirement relating to any one or combination of components illustrated in the exemplary operating environment.

The system and method of the present invention can be operational with numerous other general purpose or special purpose computing system environments or configurations. Examples of well known computing systems, environments, and/or configurations that can be suitable for use with the system and method comprise, but
are not limited to, personal computers, server computers, laptop devices, and multiprocessor systems. Additional examples comprise set top boxes, programmable consumer electronics, network PCs, minicomputers, mainframe computers, and distributed computing environments that comprise any of the above systems or devices, and the like.

In another aspect, the system and method of the present invention can be described in the general context of computer instructions, such as program modules, being executed by a computer. Generally, program modules comprise routines, programs, objects, components, data structures, etc. that perform particular tasks or implement particular abstract data types. The system and method of the present invention can also be practiced in distributed computing environments where tasks are performed by remote processing devices that are linked through a communications network. In a distributed computing environment, program modules can be located in both local and remote computer storage media including memory storage devices.

Further, one skilled in the art will appreciate that the system and method disclosed herein can be implemented via a general-purpose computing device in the form of a computer 101. The components of the computer 101 can comprise, but are not limited to, one or more processors or processing units 103, a system memory 112, and a system bus 113 that couples various system components including the processor 103 to the system memory 112.

The system bus 113 represents one or more of several possible types of bus structures, including a memory bus or memory controller, a peripheral bus, an accelerated graphics port, and a processor or local bus using any of a variety of bus architectures. By way of example, such architectures can comprise an Industry Standard Architecture (ISA) bus, a Micro Channel Architecture (MCA) bus, an Enhanced ISA (EISA) bus, a Video Electronics Standards Association (VESA) local bus, an Accelerated Graphics Port (AGP) bus, and a Peripheral Component Interconnects (PCI) bus also known as a Mezzanine bus. The bus 113, and all buses specified in this description can also be implemented over a wired or wireless network connection and each of the subsystems, including the processor 103, a mass storage device 104, an operating system 105, cardiac contractility analysis software 106, contractility data 107, a network adapter 108, system memory 112, an Input/Output Interface 110, a display adapter 109, a display device 1.11, and a human machine interface 102, can be contained within one or more remote computing devices.
114a,b,c at physically separate locations, connected through buses of this form, in effect implementing a fully distributed system. The present invention provides contractility analysis software 106 that can process contractility data received by the computer. This software can perform the calculations for ventricular shortening fraction, change in ventricular area, atrial shortening fraction, change in atrial area, change in intensity in a ventricular or atrial region or any other calculation associated with measuring cardiac contractility and changes in same. This software can also compare the cardiac contractility data for a zebrafish contacted with a test agent and a zebrafish not contacted with a test agent to determine if the agent modulates cardiac contractility. Once the agent is classified as an agent that modulates cardiac contractility, it can be further classified as a positive inotropic agent or a negative inotropic agent.

The computer 101 typically comprises a variety of computer readable media. Exemplary readable media can be any available media that is accessible by the computer 101 and comprises, for example and not meant to be limiting, both volatile and non-volatile media, removable and non-removable media. The system memory 112 comprises computer readable media in the form of volatile memory, such as random access memory (RAM), and/or non-volatile memory, such as read only memory (ROM). The system memory 112 typically contains data such as contractility data 107 and/or program modules such as operating system 105 and cardiac contractility analysis software 106 that are immediately accessible to and/or are presently operated on by the processing unit 103.

In another aspect, the computer 101 can also comprise other removable/non-removable, volatile/non-volatile computer storage media. By way of example, Figure 1 illustrates a mass storage device 104 which can provide non-volatile storage of computer code, computer readable instructions, data structures, program modules, and other data for the computer 101. For example and not meant to be limiting, a mass storage device 104 can be a hard disk, a removable magnetic disk, a removable optical disk, magnetic cassettes or other magnetic storage devices, flash memory cards, CD-ROM, digital versatile disks (DVD) or other optical storage, random access memories (RAM), read only memories (ROM), electrically erasable programmable read-only memory (EEPROM), and the like.

Optionally, any number of program modules can be stored on the mass storage device 104, including by way of example, an operating system 105 and cardiac
contractility analysis software 106. Each of the operating system 105 and cardiac contractility analysis software 106 (or some combination thereof) can comprise elements of the programming and the cardiac contractility analysis software 106. Contractility data 107 can also be stored on the mass storage device 104.

Contractility data 107 can be stored in any of one or more databases known in the art. Examples of such databases comprise, DB2®, Microsoft® Access, Microsoft® SQL Server, Oracle®, mySQL, PostgreSQL, and the like. The databases can be centralized or distributed across multiple systems. The contractility data can be ventricular length measurements, atrial length measurements, ventricular shortening fraction, atrial shortening fraction, ventricular area measurements, atrial area measurements, change in ventricular area, change in atrial area, intensity data collected at the diastole of a heartbeat, intensity data collected at the systole of a heartbeat, fluorescence data collected at the diastole of a heartbeat, fluorescence data collected at the systole of a heartbeat, change in fluorescence of a selected ventricular area and any other data associated with measuring and analyzing cardiac contractility.

In another aspect, the user can enter commands and information into the computer 101 via an input device (not shown). Examples of such input devices comprise, but are not limited to, a keyboard, pointing device (e.g., a "mouse"), a microphone, a joystick, a scanner, and the like. These and other input devices can be connected to the processing unit 103 via a human machine interface 102 that is coupled to the system bus 113, but can be connected by other interface and bus structures, such as a parallel port, game port, an IEEE 1394 Port (also known as a Firewire port), a serial port, or a universal serial bus (USB).

In yet another aspect of the present invention, a display device 111 can also be connected to the system bus 113 via an interface, such as a display adapter 109. It is contemplated that the computer 101 can have more than one display adapter 109 and the computer 101 can have more than one display device 111. For example, a display device can be a monitor, an LCD (Liquid Crystal Display), or a projector. In addition to the display device 111, other output peripheral devices can comprise components such as speakers (not shown) and a printer (not shown) which can be connected to the computer 101 via Input/Output Interface 110.

The computer 101 can operate in a networked environment using logical connections to one or more remote computing devices 114a,b,c. By way of example, a remote computing device can be a personal computer, portable computer, a server, a
router, a network computer, a peer device or other common network node, and so on. Logical connections between the computer 101 and a remote computing device 114a,b,c can be made via a local area network (LAN) and a general wide area network (WAN). Such network connections can be through a network adapter 108. A network adapter 108 can be implemented in both wired and wireless environments. Such networking environments are conventional and commonplace in offices, enterprise-wide computer networks, intranets, and the Internet 115.

For purposes of illustration, application programs and other executable program components such as the operating system 105 are illustrated herein as discrete blocks, although it is recognized that such programs and components reside at various times in different storage components of the computing device 101, and are executed by the data processors) of the computer. An implementation of cardiac contractility analysis software 106 can be stored on or-transmitted across some form of computer readable media. Computer readable media can be any available media that can be accessed by a computer. By way of example and not meant to be limiting, computer readable media can comprise "computer storage media" and "communications media." "Computer storage media" comprise volatile and non-volatile, removable and non-removable media implemented in any method or technology for storage of information such as computer readable instructions, data structures, program modules, or other data. Exemplary computer storage media comprises, but is not limited to, RAM, ROM, EEPROM, flash memory or other memory technology, CD-ROM, digital versatile disks (DVD) or other optical storage, magnetic cassettes, magnetic tape, magnetic disk storage or other magnetic storage devices, or any other medium which can be used to store the desired information and which can be accessed by a computer.

The methods and systems of the present invention can employ Artificial Intelligence techniques such as machine learning and iterative learning. Examples of such techniques include, but are not limited to, expert systems, case based reasoning, Bayesian networks, behavior based AI, neural networks, fuzzy systems, evolutionary computation (e.g. genetic algorithms), swarm intelligence (e.g. ant algorithms), and hybrid intelligent systems (e.g. Expert inference rules generated through artificial neural network or production rules from statistical learning).

The processing of the disclosed system and method of the present invention can be performed by software components. The disclosed system and method can be
described in the general context of computer-executable instructions, such as program
modules, being executed by one or more computers or other devices. Generally, program modules comprise computer code, routines, programs, objects, components, data structures, etc. that perform particular tasks or implement particular abstract data
types. The disclosed method can also be practiced in grid-based and distributed computing environments where tasks are performed by remote processing devices that are linked through a communications network. In a distributed computing environment, program modules can be located in both local and remote computer storage media including memory storage devices.

The effects of an agent, if any, can be dose dependent. For example, some agents will increase cardiac contractility at very low dosages. This will be indicative to one of skill in the art that the agent has a very highly positive inotropic potential. Other agents may not affect cardiac contractility at any dosage level. One of skill would recognize this agent as an agent with lowered or no potential to affect cardiac contractility. In another example, an agent may increase cardiac contractility at lower dosages and decrease cardiac contractility at higher dosages. One of skill in the art would recognize that this agent has negative inotropic potential at higher dosages, but has positive inotropic potential at lower dosages. Therefore, one of skill in the art can determine a dosage range for this agent that does not result in increased cardiac contractility for a subject, by categorizing the agent as an agent that can be administered above a certain dosage level, i.e. a threshold level, for increased cardiac contractility. Similarly, one of skill in the art can determine a dosage range for this agent that does not result in decreased cardiac contractility for a subject, by categorizing the agent as an agent that can be administered below a certain dosage level, i.e. a threshold level, for decreased cardiac contractility. One of skill in the art can also establish dosage levels for this agent that allow the agent to be used as a therapeutic agent for administration to a subject in need of cardiac contractility modulation, depending on whether an increase or a decrease in cardiac contractility is desired.

In another example, it is also possible for an agent to increase cardiac contractility in one zebrafish and have no effect on cardiac contractility in another zebrafish at the same dosage level. This is consistent with humans, where only some patients develop increased cardiac contractility in response to drugs with negative cardiac effects. In this instance, it would be clear to one of skill in the art that the
agent may increase cardiac contractility for some individuals at a certain dosage level. One of skill in the art could then increase the dosage to determine if upon increasing the dosage of the agent more zebrafish exhibit an increase in cardiac contractility. One of skill in the art could also decrease the dosage to determine if upon decreasing the dosage of the agent, fewer zebrafish exhibit an increase in cardiac contractility. This would allow one of skill in the art to establish a dosage range for the agent that would decrease the incidence of increased cardiac contractility across a population of zebrafish. These results can be extrapolated to establish a dosage range for the agent that would reduce the incidence of increased cardiac contractility across a population of other subjects, such as humans.

Similarly, it is also possible for an agent to decrease cardiac contractility in one zebrafish and have no effect on cardiac contractility in another zebrafish at the same dosage level. This is consistent with humans, where only some patients develop decreased cardiac contractility in response to drugs with negative cardiac effects. In this instance, it would be clear to one of skill in the art that the agent may decrease cardiac contractility for some individuals at a certain dosage level. One of skill in the art could then increase the dosage to determine if upon increasing the dosage of the agent more zebrafish exhibit a decrease in cardiac contractility. One of skill in the art could also decrease the dosage to determine if upon decreasing the dosage of the agent, fewer zebrafish exhibit a decrease in cardiac contractility. This would allow one of skill in the art to establish a dosage range for the agent that would reduce the incidence of decreased cardiac contractility across a population of zebrafish. These results can be extrapolated to establish a dosage range for the agent that would reduce the incidence of decreased cardiac contractility across a population of other subjects, such as humans.

Any of the agents identified as increasing or decreasing cardiac contractility utilizing the methods of the present invention can be administered to another model for detection of increased or decreased cardiac contractility, such as a mouse model, a rat model etc. to determine if the agent produces similar effects in a non-zebrafish model. Ultimately, it is possible to use any of the threshold levels or dosage ranges established using the methods of the present invention to establish dosage ranges for administration to any subject, for example, a mammal, preferably human, and can include, but is not limited to mouse, rat, cow, guinea pig, hamster, rabbit, cat, dog, goat, sheep, monkey, horse and chimpanzee. Therefore, dosage ranges for other
subjects, such as humans, can be extrapolated from the dosage ranges obtained utilizing the methods of the present invention. Furthermore, the results obtained utilizing the methods of the present invention can be used to predict whether an agent, for example, a drug, would have the same effect in humans. Once an agent is identified as increasing cardiac contractility utilizing the methods set forth herein, this agent can be classified as an agent that could increase cardiac contractility in humans. Similarly, once an agent is identified as decreasing cardiac contractility utilizing the methods set forth herein, this agent can be classified as an agent that could decrease cardiac contractility in humans. Therefore, the methods of the present invention can also include correlating the effect of the agent on the zebrafish with a predicted effect of the agent on a mammal, for example, a human. This information identifying the effect of the agent as a possible or predicted effect of the agent in a mammal can be provided to a company, the government, a health care provider, a patient etc.

As mentioned above, agents identified utilizing the methods of the present invention can be used to modulate cardiac contractility therapeutically. Therefore, the agents identified by the methods of the present invention can also be placed in a suitably acceptable pharmaceutical carrier for administration to a subject utilizing appropriate dosage levels. The subject can be any mammal, preferably human, and can include, but is not limited to mouse, rat, cow, guinea pig, hamster, rabbit, cat, dog, goat, sheep, monkey, horse and chimpanzee. By pharmaceutically acceptable is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the selected agent without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. In addition, one can include other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc.

The compounds identified via the methods of the present invention can be administered to a subject via oral administration, nebulization, inhalation, mucosal administration, intranasal administration, intratracheal administration, intravenous administration, intraperitoneal administration, subcutaneous administration, intracerebral delivery (such as intracerebral injection or by convection enhanced delivery) and intramuscular administration.

Therefore, the methods set forth herein provide an efficient screening strategy that can save the pharmaceutical industry considerable time, money and effort by
identifying drugs early in development that could cause cardiac abnormalities in humans. Furthermore, the methods set forth herein can be utilized to identify inotropic agents for any purpose, including identifying inotropic agents that have deleterious effects as well as identifying inotropic agents that have desirable or therapeutic effects. For example, agents with desired inotropic effects can be identified and administered to subjects in need of cardiac contractility modulation. These agents can be negative inotropic agents that decrease cardiac contractility and can be used to treat hypertension or any other condition where it is desirable to decrease cardiac contractility. Positive inotropic agents can also be identified and administered to subjects, for example, to treat shock or any other condition where it is desirable to increase cardiac contractility. Any of the therapeutic agents identified utilizing the methods set forth herein can be administered in combination with one or more therapeutic agents such as, but not limited to, agents that modulate blood pressure (for example, ACE inhibitors, alpha agonists, alpha blockers, angiotensin II receptor blockers, diuretics), antiarrhythmics, cholesterol lowering agents, blood thinners, rate control agents, or vasodilators.

EXAMPLE I

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

Transgenic zebrafish with heart-specific expression of green reef coral fluorescent protein (GRCFP) were used to test the effect of drugs with known torsadogenic potential on the rhythmicity of zebrafish heartbeat patterns. Heart rate patterns generated from video streams of beating zebrafish hearts were analyzed to determine if the heartbeat pattern of a particular zebrafish embryo is regular or irregular. Moreover, it was found that torsadogenic drugs cause irregular heartbeat
patterns in zebrafish and that non-torsadogenic drugs do not cause a loss of cardiac rhythmicity in zebrafish. TG(cMLC2:GRCFP) zebrafish are the first high-throughput in vivo model for directly assessing drugs for torsadogenic potential.

The methods can be applied to in vivo heart rate measurements in, for example, a transgenic zebrafish that specifically expresses green reef coral fluorescent protein (GRCFP) in the heart under control of the cardiac myosin light chain-2 promoter (cMLC-2). Video streams of beating fluorescent zebrafish hearts can be used to generate heart rate plots. These heart rate plots can be analyzed for drug-induced irregular heartbeat patterns. This invention shows that the presence of drug-induced irregular heartbeat pattern is a predictor of the potential of a drug to cause torsadogenic potential (TdP) than heart rate alone.

Zebrafish embryos at 2 days post fertilization (dpf) were soaked overnight in drugs at concentrations of 1, 10, and 100 µM. DMSO was used as a vehicle control. The drugs were classified as follows, based on their propensity to cause torsadogenic potential (TdP) in humans:

**Category 1**-Drugs withdrawn from the US market due to unacceptable risk of TdP: astemizole, cisapride, terfenadine

**Category 2**-Drugs that carry significant risk of TdP: haloperidol, pimozide, bepridil

**Category 3**-Drugs with little to no association with TdP: propafenone, ketoconazole, desipramine, fluoxetine

Video streams of the beating zebrafish hearts were collected at 3dpf. The heart rates of the 3dpf zebrafish treated with drugs were automatically calculated from these video streams, and heart rate plots were generated based on the change in fluorescence intensity of the heart during each beat. These plots are graphical representations of the zebrafish heartbeat pattern.

All of the drugs tested can lower heart rate in zebrafish compared to vehicle control, but not all drugs which lower heart rate cause an irregular heart beat pattern. Normal 3dpf zebrafish heart rates are between 160-180 beats/min. To determine if
the TG(cMLC2:GRCFP) zebrafish can be used to predict torsadogenic potential of
drugs, heart rate plots from zebrafish with heart rates that were lower than 90
beats/min were analyzed. The pattern was scored as arrhythmic if any of the
following standards were met:

1. Plot has a non-repeating pattern of contractions and relaxations.
2. Magnitude of the change in fluorescence intensity of any heartbeat (peak or
trough) is at least 30% different (greater or smaller) than the average heartbeat
for that plot.
3. Time between peaks (τ; relaxation phase) or the time between troughs
(τ'; contraction phase) is at least 30% different (longer or shorter) than the
average for that plot.

Blinded analyses of heart rate readouts were performed independently by 3
different scientists. All 3 analyses successfully identified at least one irregular heart
rate plot in 6/6 torsadogenic drug-treated groups. There was good agreement among
the scientists as to which plots showed irregular patterns. Not all of the torsadogenic
drug-treated zebrafish showed irregular heartbeat patterns. This is consistent with
humans, where only some patients develop arrhythmia in response to drugs with toxic
cardiac effects.

None of the analyses identified non-torsadogenic drug as causing irregular
heartbeat patterns in zebrafish (Table 1). Table 1 contains the blinded analyses of
heart rate plots for rhythmicity that were independently performed by 3 different
scientists. Heart rate plots were generated from zebrafish treated with compounds
with varying degrees of torsadogenic risk (shown on left). Data are shown as
irregular heart rate patterns detected/total analyzed for each treatment group. All 3
analyses successfully identified at least one irregular heart rate pattern in 6/6
torsadogenic drug-treated groups, while 0/4 non-torsadogenic drugs caused a loss of
cardiac rhythmicity.

The heart rate patterns that were used in the analyses are provided in FIGS. 5
through 49. The determination of rhythmic vs arrhythmic from Analysis 1 is included.

**EXAMPLE II**

Transgenic zebrafish expressing green reef coral fluorescent protein (GRCFP)
specifically in the heart were used to quantify changes in contractility induced by
treatment with isoproterenol, a positive inotropic drug (increases contractility) and verapamil, a negative inotropic drug (decreases contractility). Fluorescent reporter expression in the heart allows easy visualization of the heart and clear discernment of the ventricle and the atrium, because the ventricle has brighter fluorescence. However, as mentioned above, the methods described herein can also be performed with nontransgenic zebrafish that do not express a fluorescent reporter protein in the heart.

Zebrafish at 2dpf were treated with either 100 µM isoproterenol, a positive inotropic drug, or 10 µM verapamil, a negative inotropic drug. At 3dpf, video streams of the zebrafish were collected using the Discovery-1 high-content imaging system with a 2x objective. Using MetaMorph software, a line was drawn along the length of the ventricle (longest diameter) at the end of diastole and at the end of systole. The length of the lines were measured and used to calculate ventricular shortening fraction. Ventricular shortening fraction was calculated as:

\[
\frac{\text{End diastolic ventricular length} - \text{End systolic ventricular length}}{\text{End diastolic ventricular length}}
\]

Similar to the effects of these drugs in humans, it was found that isoproterenol significantly increased ventricular shortening fraction, while verapamil significantly decreased ventricular shortening fraction in zebrafish compared to negative controls (Fig. 2). These results indicate that zebrafish are a good model for measuring cardiac contractility.

Contractility can be measured in alternative ways in zebrafish with fluorescent hearts. The change in ventricular area during each heartbeat can be used to determine contractility. To measure the change in total area of the ventricle, image thresholding can be applied using MetaMorph software to video streams of 3dpf drug-treated zebrafish, such that only the ventricle is highlighted. This is possible because the fluorescent protein is more highly expressed in the ventricle than in the atrium. The area of the ventricle is measured by the software, and recorded at the end of diastole and at the end of systole. The percent change in ventricular area can be calculated using the following equation:
End diastolic ventricular area — End systolic ventricular area \( \chi \frac{100}{100} \)

End diastolic ventricular area

In addition to morphological measurements, cardiac contractility can be assessed by choosing a region of interest within the ventricle and measuring the change in fluorescence intensity of that region with each heartbeat. A larger change in fluorescence intensity would correspond to a larger contractility. For measuring contractility, the fluorescence intensity of a specified region of interest at the end of diastole and at the end of systole from each heartbeat can be determined via standard methods known in the art, including microscopy and analysis of fluorescence data obtained from automatically generated heartbeat plots. Furthermore, the cardiac contractility measurements set forth herein can be combined with assessments or measurements of other cardiac functions. For example, if after administering a test agent, one of skill in the art observes an increase in the change in fluorescence intensity of a ventricular region as compared to a control zebrafish and also observes, either visually or via other means, that a heartbeat plot from which the fluorescence data is obtained corresponds to an arrhythmic heartbeat plot, one of skill in the art can classify the agent as an agent that increases cardiac contractility and causes arrhythmia.

Transgenic zebrafish expressing green reef coral fluorescent protein specifically in the heart were used to show that isoproterenol and verapamil, drugs that change contractility in humans, also affect contractility in zebrafish. Automated image acquisition of beating hearts with measurement of VSF was combined for these studies. The present invention is not limited to measuring cardiac contractility by measuring VSF as it also provides measurement of ventricular area changes, measurement of atrial area changes, measurement of atrial shortening factor, changes in intensity of a region within the ventricle or atrium during each heartbeat to assess cardiac contractility. Zebrafish can therefore be used as an assay model for determining the effects of drugs on contractility. These \textit{in vivo} assays will allow toxicity testing of drugs early in pre-clinical development in a high-throughput manner, thus providing an advantage over current mammalian models for contractility assessment. These assays will also allow identification of therapeutic agents for modulation of cardiac contractility.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by
reference into this application in order to more fully describe the state of the art to which this invention pertains.

While this invention has been described in connection with preferred embodiments and specific examples, it is not intended that the scope of the invention be limited to the particular embodiments set forth, as the embodiments herein are intended in all respects to be illustrative rather than restrictive.

Unless otherwise expressly stated, it is in no way intended that any method set forth herein be construed as requiring that its steps be performed in a specific order. Accordingly, where a method claim does not actually recite an order to be followed by its steps or it is not otherwise specifically stated in the claims or descriptions that the steps are to be limited to a specific order, it is no way intended that an order be inferred, in any respect. This holds for any possible non-express basis for

<table>
<thead>
<tr>
<th>Drug</th>
<th>Scientist 1</th>
<th>Scientist 2</th>
<th>Scientist 3</th>
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<tr>
<td><strong>Withdrawn from market</strong></td>
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<tr>
<td>Astemizole</td>
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<tr>
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<td>5/5</td>
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<tr>
<td>Terfenadine</td>
<td>7/8</td>
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<tr>
<td>Haloperidol</td>
<td>2/4</td>
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</tr>
<tr>
<td>Pimozide</td>
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<td>1/2</td>
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While this invention has been described in connection with preferred embodiments and specific examples, it is not intended that the scope of the invention be limited to the particular embodiments set forth, as the embodiments herein are intended in all respects to be illustrative rather than restrictive.

Unless otherwise expressly stated, it is in no way intended that any method set forth herein be construed as requiring that its steps be performed in a specific order.
interpretation, including: matters of logic with respect to arrangement of steps or operational flow; plain meaning derived from grammatical organization or punctuation; the number or type of embodiments described in the specification.

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.
References


What is claimed is:

1. A method of classifying a heart rate pattern as arrhythmic comprising:
   a) obtaining a heart rate pattern having at least one peak and at least one trough from a transgenic zebrafish that expresses a fluorescent reporter protein in the heart;
   b) determining at least one of the following:
      i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations;
      ii) if a magnitude of change in a fluorescence intensity of a heartbeat (peak or trough) is a first threshold greater or smaller than an average heartbeat for the pattern;
      iii) if a time between peaks (t; relaxation phase) or a time between troughs (f; contraction phase) is at least a second threshold different than an average time between troughs for the pattern; and
   c) classifying the heart rate pattern as arrhythmic if any one of the determinations of b) is true.

2. The method of claim 1, wherein the first threshold is at least about 5%.

3. The method of claim 1, wherein the second threshold is at least about 5%.

4. The method of claim 1, wherein the second threshold is longer than the average time between troughs for the pattern.

5. The method of claim 1, wherein the second threshold is shorter than the average time between troughs for the pattern.

6. The method of claim 1, wherein the fluorescent reporter protein is under the control of the cardiac myosin light chain-2 promoter (cMLC-2).

7. The method of claim 1, wherein the fluorescent reporter protein green reef coral fluorescent protein (GRCFP).

8. A method for identifying an agent that causes arrhythmia comprising:
   a. contacting a transgenic zebrafish that expresses a fluorescent reporter protein in the heart with a test agent;
b. obtaining a heart rate pattern having at least one peak and at least one trough from the zebrafish contacted with the test compound;
c. determining one of the following:
   i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations;
   ii) if a magnitude of change in a fluorescence intensity of a heartbeat (peak or trough) is a first threshold greater or smaller than an average heartbeat for the pattern;
   iii) determining if a time between peaks ($t$; relaxation phase) or a time between troughs ($t'$; contraction phase) is at least a second threshold different than an average time between troughs for the pattern; and

d) classifying the heart rate pattern as arrhythmic if any of the determinations of c) is true, such that if the heart rate pattern is classified as arrhythmic, the test agent is an agent that causes arrhythmia.

9. The method of claim 8, wherein the first threshold is at least about 5%.
10. The method of claim 8, wherein the second threshold is at least about 5%.
11. The method of claim 8, wherein the second threshold is longer than the average time between troughs for the pattern.
12. The method of claim 8, wherein the second threshold is shorter than the average time between troughs for the pattern.
13. The method of claim 8, wherein the fluorescent reporter protein is under the control of the cardiac myosin light chain-2 promoter (cMLC-2).
14. The method of claim 8, wherein the fluorescent reporter protein green reef coral fluorescent protein (GRCFP).
15. A method of classifying a heart rate pattern as arrhythmic comprising:
   a. obtaining a heart rate pattern having at least one peak and at least one trough from a zebrafish;
   b. determining at least one of the following:
      i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations;
ii) if a magnitude of change in optical density of a region of interest within the heart (peak or trough) is a first threshold greater or smaller than an average change in optical density for the pattern;

iii) if a time between peaks (t; relaxation phase) or a time between troughs (f; contraction phase) is at least a second threshold different than an average time between troughs for the pattern; and

c. classifying the heart rate pattern as arrhythmic if any one of the determinations of b) is true.

16. The method of claim 15, wherein the second threshold is at least about 5%.

17. The method of claim 15, wherein the second threshold is longer than the average time between troughs for the pattern.

18. The method of claim 15, wherein the second threshold is shorter than the average time between troughs for the pattern.

19. A method for identifying an agent that causes arrhythmia comprising:

a. contacting a zebrafish with a test agent;

b. obtaining a heart rate pattern having at least one peak and at least one trough from the zebrafish contacted with the test agent;

c. determining at least one of the following:

i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations;

ii) if a magnitude of change in optical density of a region of interest within the heart (peak or trough) is a first threshold greater or smaller than an average change in optical density for the pattern;

iii) if a time between peaks (t; relaxation phase) or a time between troughs (f; contraction phase) is at least a second threshold different than an average time between troughs for the pattern; and

d. classifying the heart rate pattern as arrhythmic if any one of the determinations of c) is true, such that if the heart rate pattern is classified as arrhythmic, the test agent is an agent that causes arrhythmia.
20. The method of claim 19, wherein the second threshold is at least about 5%.

21. The method of claim 19, wherein the second threshold is longer than the average time between troughs for the pattern.

22. The method of claim 19, wherein the second threshold is shorter than the average time between troughs for the pattern.

23. A method of classifying a heart rate pattern as arrhythmic comprising:
   a. obtaining a heart rate pattern having at least one peak and at least one trough from a zebrafish;
   b. determining at least one of the following:
      i) if the heart rate pattern has a non-repeating pattern of contractions and relaxations;
      ii) if a magnitude of change in a morphological characteristic (peak or trough) is a first threshold greater or smaller than an average change in a morphological characteristic for the pattern;
      iv) if a time between peaks (t; relaxation phase) or a time between troughs (f; contraction phase) is at least a second threshold different than an average time between troughs for the pattern; and
   c. classifying the heart rate pattern as arrhythmic if any one of the determinations of b) is true.

24. The method of claim 23, wherein the change in a morphological characteristic is a change in the length of the heart, a change in the size of the perimeter of the heart or a change in projected volume.

25. The method of claim 23, wherein the second threshold is at least about 5%.
26. The method of claim 23, wherein the second threshold is longer than the average time between troughs for the pattern.

27. The method of claim 23, wherein the second threshold is shorter than the average time between troughs for the pattern.

28. A system for classifying a heart pattern as arrhythmic comprising a heart pattern data receiving unit and a heart pattern data classifying unit.

29. A method of identifying an agent that modulates cardiac contractility comprising:
   a. contacting a transgenic zebrafish that expresses a fluorescent protein in the heart with a test agent;
   b. measuring cardiac contractility;
   c. determining the effect of the test agent on cardiac contractility such that if there is a difference in cardiac contractility between the zebrafish contacted with the test agent and a zebrafish not contacted with the test agent, the test agent is an agent that modulates cardiac contractility.

30. The method of claim 29, wherein the difference in cardiac contractility is an increase in contractility.

31. The method of claim 29, wherein the difference in cardiac contractility is a decrease in contractility.

32. The method of claim 29, wherein expression of the fluorescent protein is under the control of a heart specific promoter.

33. The method of claim 32, wherein the promoter is the cardiac myosin light chain-2 promoter (cMLC-2).
34. The method of claim 29, wherein the fluorescent protein is green reef coral fluorescent protein (GRCFP).

35. The method of claim 29, wherein cardiac contractility is measured by calculating ventricular shortening fraction.

36. The method of claim 35, wherein ventricular shortening fraction is calculated according to the following equation:

\[
\frac{\text{End diastolic ventricular length} - \text{End systolic ventricular length}}{\text{End diastolic ventricular length}}
\]

37. The method of claim 29, wherein cardiac contractility is measured by calculating the percent change in ventricular area during each heartbeat.

38. The method of claim 37, wherein the percent change in ventricular area is calculated according to the following equation:

\[
\frac{\text{End diastolic ventricular area} - \text{End systolic ventricular area}}{\text{End diastolic ventricular area}} \times JQQ
\]

39. The method of claim 29 or 38, wherein cardiac contractility is measured by measuring the change in intensity of a selected region within a ventricle for a heartbeat.

40. The method of claim 39, wherein the change in intensity is calculated by calculating the difference between phase contrast gray intensity of a region within a ventricle at the end of the diastole and phase contrast gray intensity of the region within the ventricle at the end of the systole for a heartbeat.
41. The method of claim 40, wherein the difference is an increase in intensity in the zebrafish contacted with the test agent as compared to a zebrafish not contacted with the test agent.

42. The method of claim 40, wherein the difference is a decrease in intensity in the zebrafish contacted with the test agent as compared to a zebrafish not contacted with the test agent.

43. The method of claim 40, wherein intensity at the end of the diastole and intensity at the end of the systole for a heartbeat is obtained from a heartbeat plot.

44. The method of claim 39, wherein cardiac contractility is measured by measuring the change in fluorescence intensity of a selected region within a ventricle for a heartbeat.

45. The method of claim 44, wherein the change in fluorescence intensity is calculated by calculating the difference between fluorescence intensity of a region within a ventricle at the end of the diastole and fluorescence intensity of the region within the ventricle at the end of the systole for a heartbeat.

46. The method of claim 45, wherein the difference is an increase in fluorescence intensity in the zebrafish contacted with the test agent as compared to a zebrafish not contacted with the test agent.

47. The method of claim 45, wherein the difference is a decrease in fluorescence intensity in the zebrafish contacted with the test agent as compared to a zebrafish not contacted with the test agent.

48. The method of claim 45, wherein fluorescence intensity at the end of the diastole and fluorescence intensity at the end of the systole for a heartbeat is obtained from a heartbeat plot generated from fluorescence intensity data.
49. A method of identifying an agent that modulates cardiac contractility comprising:
   a. contacting a zebrafish with a test agent;
   b. measuring cardiac contractility by calculating the percent change in ventricular area during each heartbeat;
   c. determining the effect of the test agent on cardiac contractility such that if there is a difference in cardiac contractility between the zebrafish contacted with the test agent and a zebrafish not contacted with the test agent, the test agent is an agent that modulates cardiac contractility.

50. The method of claim 49, wherein the difference in cardiac contractility is an increase in contractility.

51. The method of claim 49, wherein the difference in cardiac contractility is a decrease in contractility.

52. The method of claim 49, wherein the percent change in ventricular area is calculated according to the following equation:

   \[
   \frac{\text{End diastolic ventricular area} - \text{End systolic ventricular area}}{\text{End diastolic ventricular area}} \times 100
   \]

53. The method of claim 49 or 52, wherein cardiac contractility is measured by measuring the change in intensity of a selected region within a ventricle for a heartbeat.

54. The method of claim 53, wherein the change in intensity is calculated by calculating the difference between phase contrast gray intensity of a region within a ventricle at the end of the diastole and phase contrast gray intensity of the region within the ventricle at the end of the systole for a heartbeat.
55. The method of claim 54, wherein the difference is an increase in intensity in the zebrafish contacted with the test agent as compared to a zebrafish not contacted with the test agent.

56. The method of claim 54, wherein the difference is a decrease in intensity in the zebrafish contacted with the test agent as compared to a zebrafish not contacted with the test agent.

57. The method of claim 54, wherein intensity at the end of the diastole and intensity at the end of the systole for a heartbeat is obtained from a heartbeat plot.
receiving a heart rate pattern having at least one peak and at least one trough

determining at least one of the following in blocks 202, 203, or 204

determining if the heart rate pattern has a non-repeating pattern of contractions and relaxations

determining if a magnitude of change in a fluorescence intensity of a heartbeat (peak or trough) is a first threshold greater or smaller than an average heartbeat for the pattern

determining if a time between peaks (t; relaxation phase) or a time between troughs (t\'; contraction phase) is at least a second threshold different (longer or shorter) than an average time between troughs for the pattern

classifying the heart rate pattern as arrhythmic if any of the determinations is true
receiving a heart rate pattern

determining a feature vector for the heart rate pattern

classifying the heart rate pattern as arrhythmic based on the feature vector using a trained classifier

FIGURE 3
FIGURE 4
Bepridil 4
Rhythmic

FIGURE 10
Pimozide 2
Arrhythmic

**FIGURE 38**
Terfenadine 5
Arrhythmic *

FIGURE 46
Terfenadine 8
Arrhythmic *

FIGURE 49