

A Sensor Based Technique for Pharmacological Safety Testing of Cardiac Transport Proteins NCX, NaKATPase and Respiratory Chain Complexes

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Cardiac Transporters in Pharmacology

Ion channels have long been targets for cardiac safety testing. However, other proteins involved in the transport of ions across membrane barriers are also relevant for pharmacological safety.

To drive the progress in pharmacological investigation of cardiac transporters we have evaluated new methodologies to study both desired and unwanted effects of compounds on several cardiac transport proteins, based on solid supported membrane (SSM) and impedance technologies, recording from either 1 or 96 wells simultaneously. As protein containing samples we have used HEK293 cell lines, mitochondrial inner membrane vesicles from pig heart as well as iPSC-derived cardiomyocytes on automated devices.

We have focused on two transporters that emerged as targets for cardiac pharmacology and safety testing. First we investigated the Sodium-Calcium Exchanger (NCX), which plays an important role in the cellular calcium homeostasis under

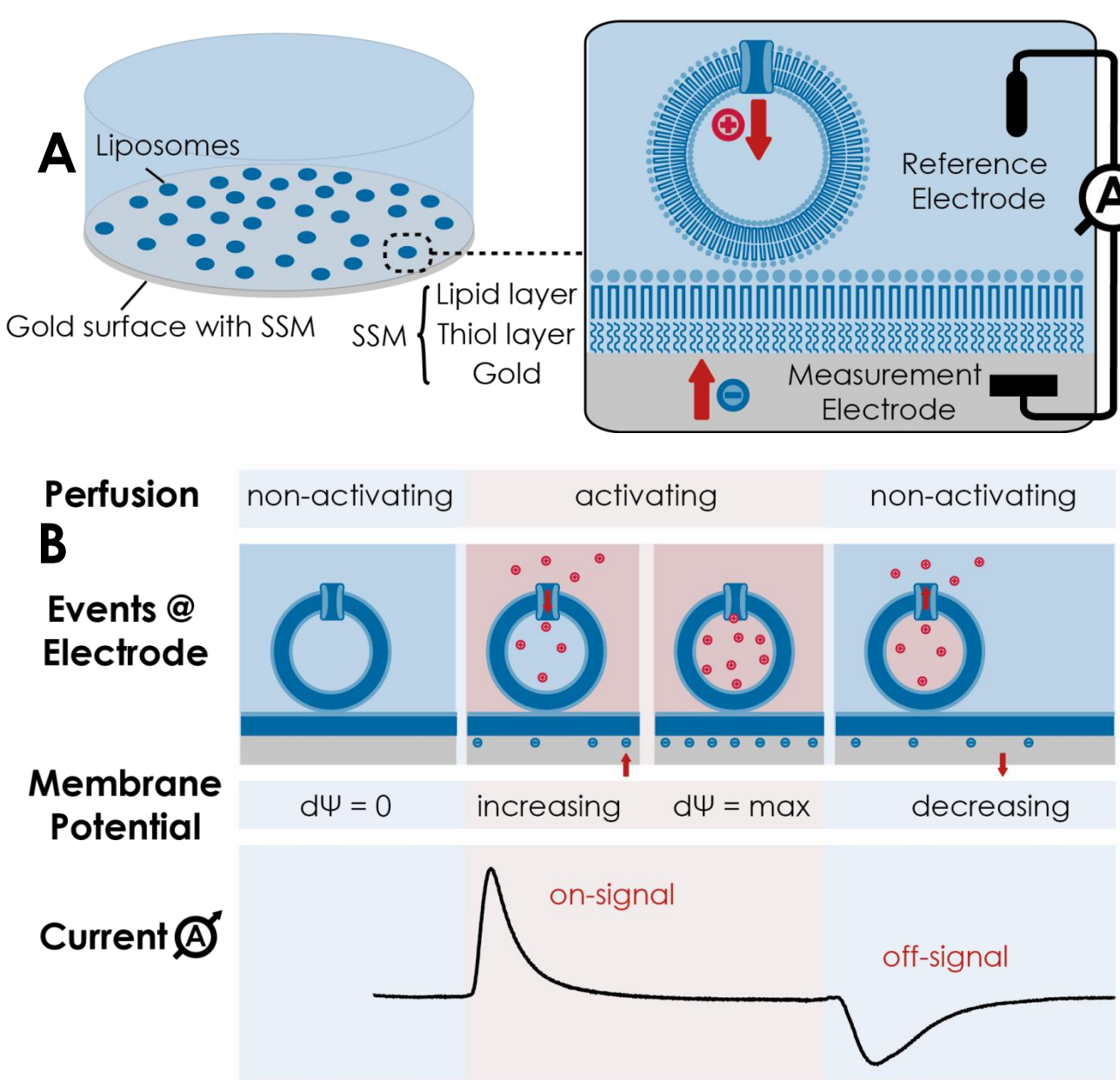
physiological and pathological conditions. Inhibition of NCX is thought to be beneficial in ischemia/reperfusion injury but also might have an anti-arrhythmic effect. As a second target we investigated Na^+/K^+ -ATPase, which plays a major role in the regulation of basic cardiac functions and is targeted by cardiac glycosides, illustrating its critical involvement in both beneficial and potentially harmful pharmacological effects. Additionally, we have investigated the complexes of the respiratory chain as interference with these ATP-producing proteins can lead to lethal side effects.

We developed several new methods to investigate these cardiac transporters in 96 well formats, thus vastly increasing throughput, a critical factor in pharmacological screening. We showed dose dependent effect of known substrates and inhibitors, thus verifying the reliability of our methods. As first result we could show that inhibition of NCX increases the beat rate of iPSC derived cardiomyocytes.

Literature

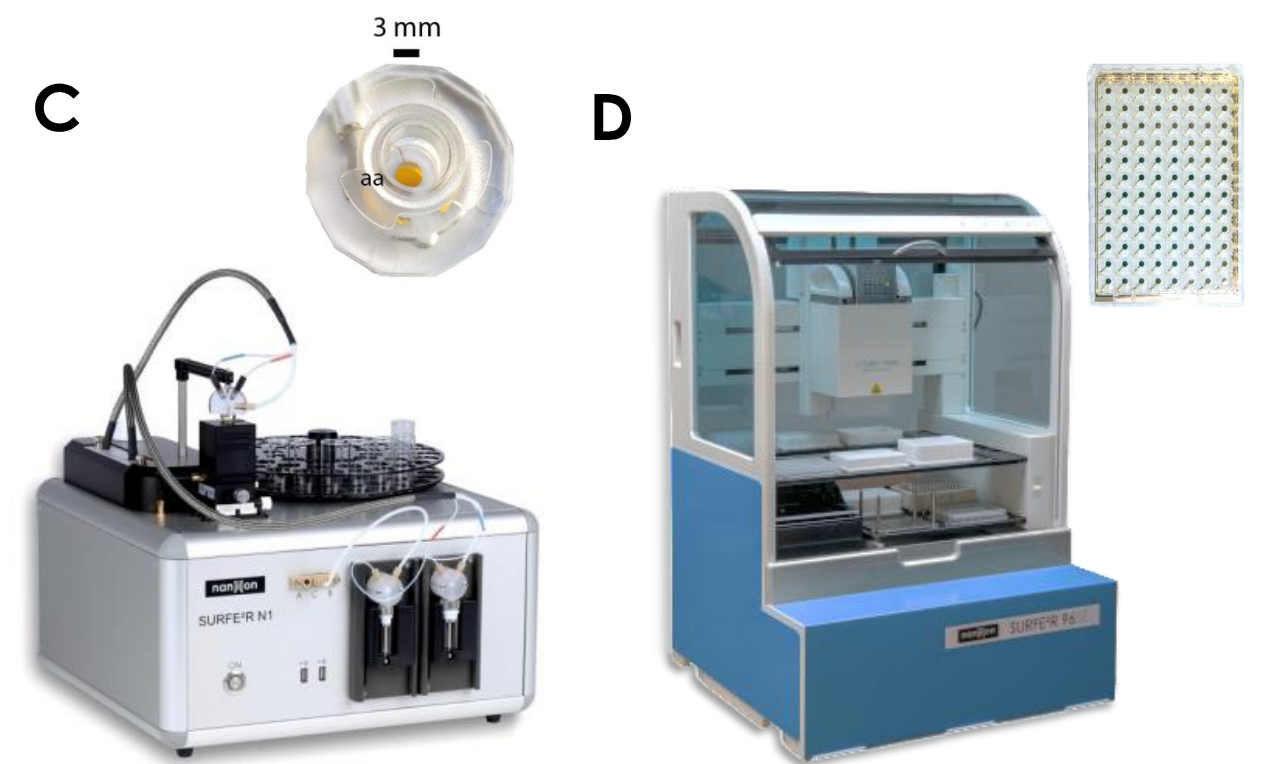
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SSM-based electrophysiology: A method to resolve electric events generated by transporters

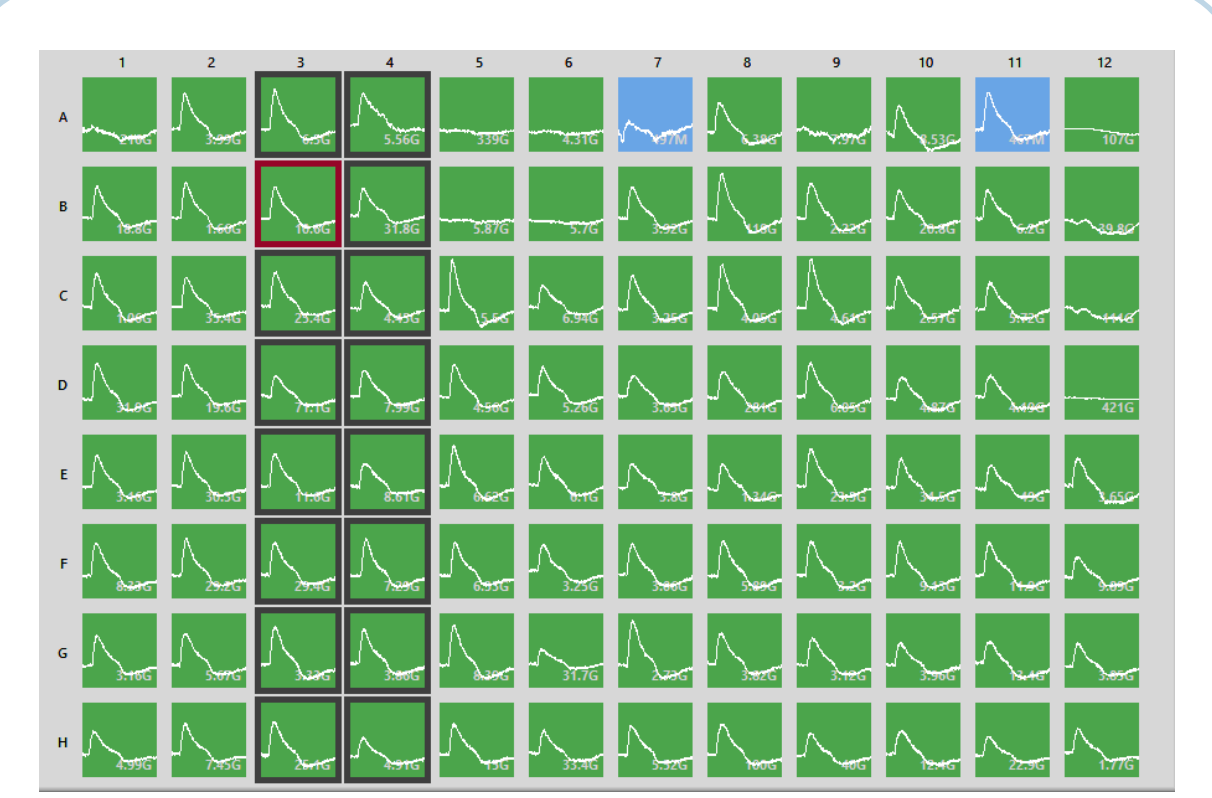
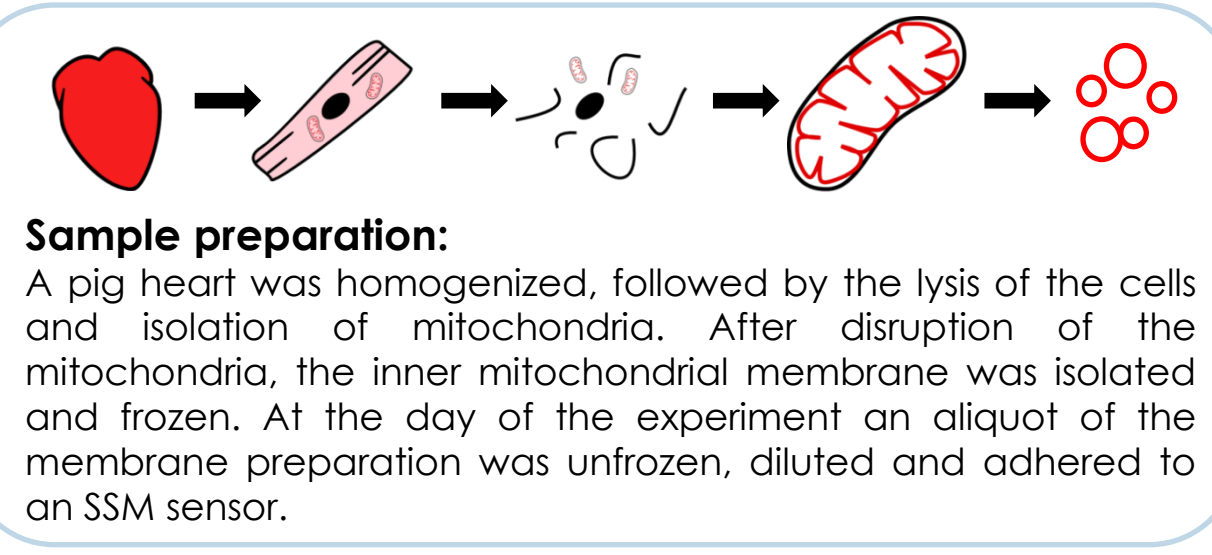


A: Structure of an SSM sensor. The 3 mm diameter thin layer gold electrode is coated with an SSM consisting of a thiol and a lipid monolayer. On top liposomes or membrane vesicles containing the target protein are adsorbed. **B:** Protocol of one activation cycle. The sensor is perfused with a passive non activating solution followed by an activating solution, containing a transported substrate or ATP. During this step a transient current occurs, since the active transporter moves charge into the vesicles and this generates a membrane potential. Instrumentation: We used the SURFER N1 (**C**) and the SURFER 96SE (**D**) instruments in this project. The respective SSM sensors are depicted above the instruments. The SURFER N1 is a single channel measurement instrument, whereas the SURFER 96SE is a robotic device measuring 96 sensors in parallel, designed for experiments that require a high throughput.

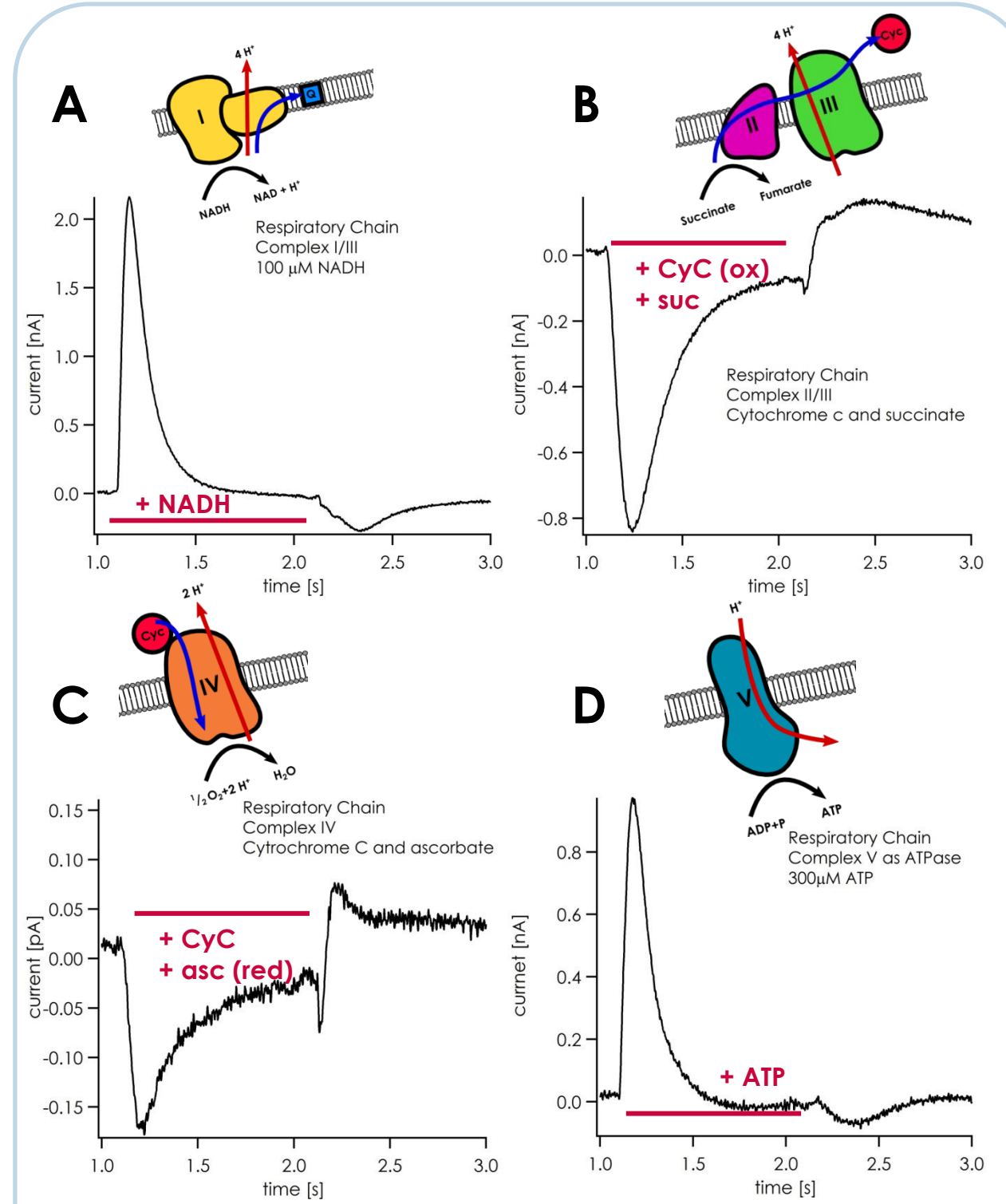
Solid Supported Membrane (SSM)-based electrophysiology is a method, which allows the resolution of low amplitude electric events in biological membranes, bringing the advantages of electrophysiology to the transporter field. It enables real-time activity measurement of electrogenic SLC-transporters, MFS-transporters and ion pumps, localized in the plasma membrane, in intracellular or bacterial membranes. Unlike in cell-based electrophysiology, a large capacitive sensor coated with a high amount of membrane vesicles or proteoliposomes is used. In this way any process that generates an electrical potential can be registered with high amplification. The method was established in the 90's and has recently been scaled-up to a 96-well format.



Direct measurement of respiratory chain complex activity in cardiac mitochondrial membranes

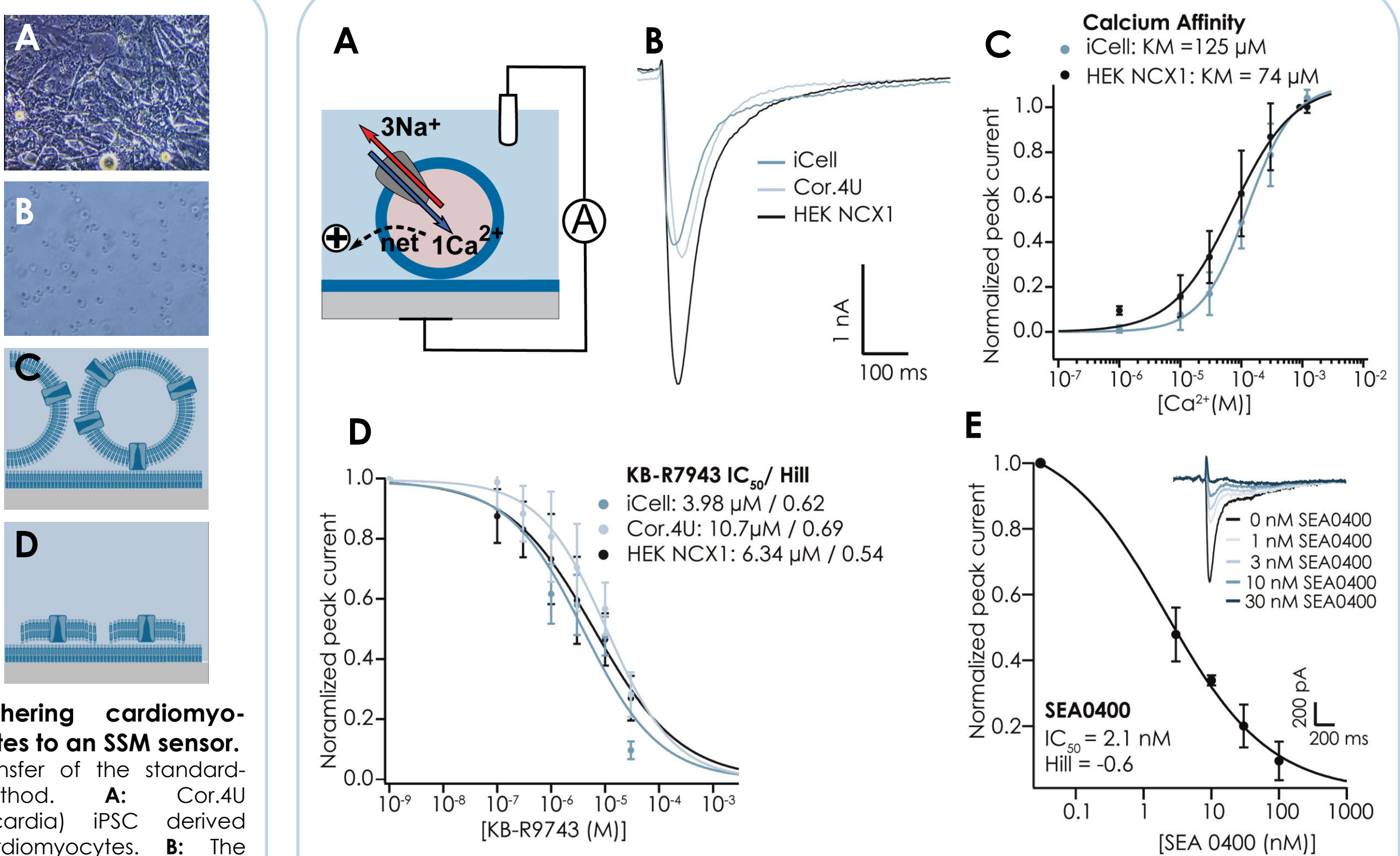


Upscaling: Activity measurement of complex I in mitochondrial membranes using the high throughput platform SURFER 96SE. 96 individual measurements allow for compound testing with high efficiency. In each well the activity of complex I can be triggered repeatedly, allowing for baseline and control measurements as well as application of different concentrations or even different compounds in one well. Each measurement takes 5 minutes before the next application can be started. This way over 1k data points are generated in one hour.



The activity of individual respiratory chain complexes recorded with SSM based electrophysiology. **A:** Oxidation of NADH leads to transport of four protons across the membrane. **B:** Oxidation of succinate: Electrons are transferred to cytochrome c via complex III while four protons move to the intermembrane space. **C:** Reduction of oxygen by consumption of electrons from cytochrome c and transport of two protons to the intermembrane space. **D:** Complex V generates ATP from ADP plus phosphate. Here it works backwards as ATPase.

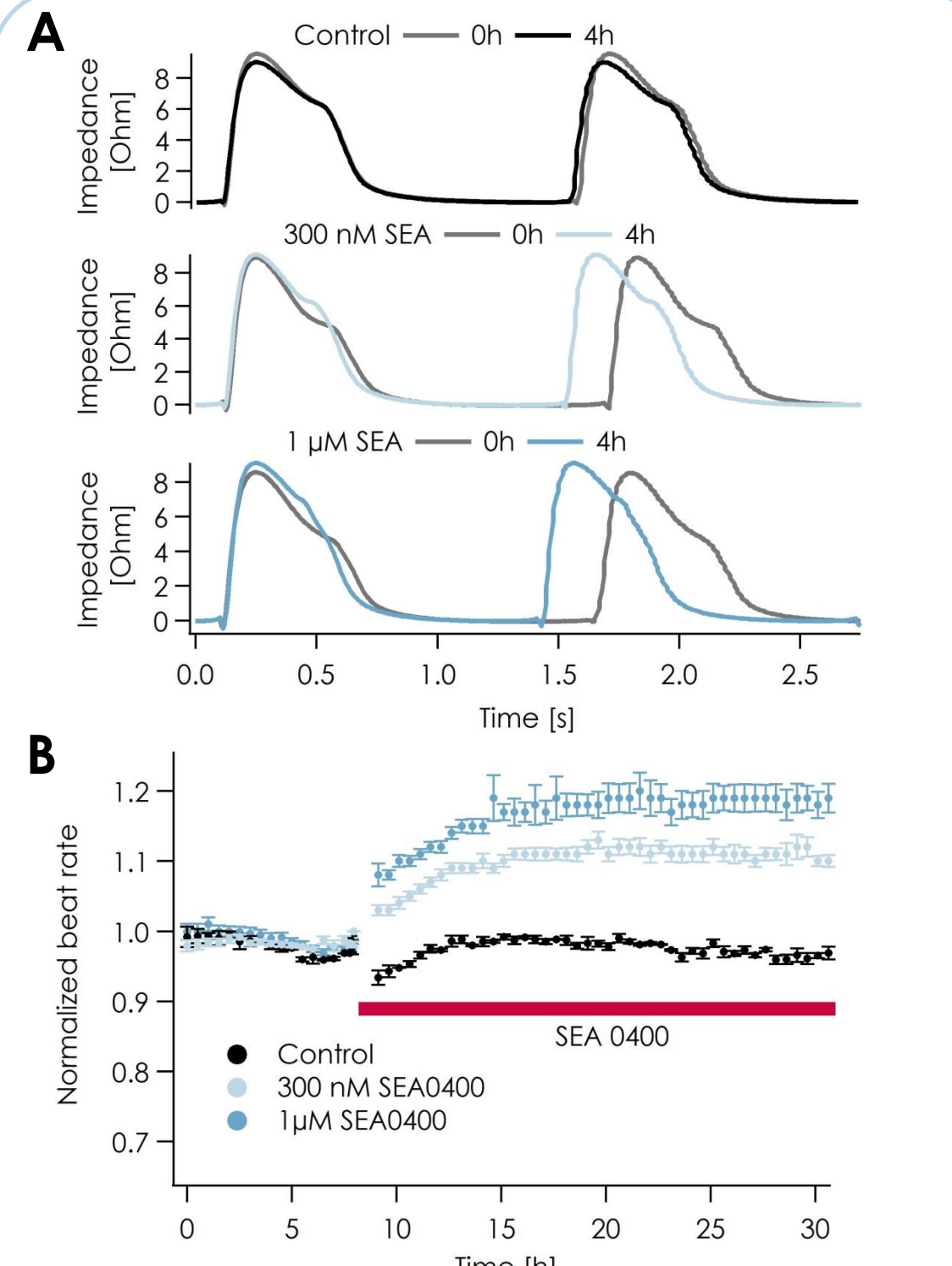
Human iPSC derived cardiomyocytes show NCX currents and inhibition of NCX increases the beat rate



Adhering cardiomyocytes to an SSM sensor. Transfer of the standard method. **A:** Cor.4U (Ncardia) iPSC derived cardiomyocytes. **B:** The cardiomyocytes were detached using trypsin. **C:** The cardiomyocytes adhere to a lipid coated SSM sensor. **D:** The sensor is rinsed in a fluidic system with high speed. This leads to tearing of the cells - only a membrane sheet remains.

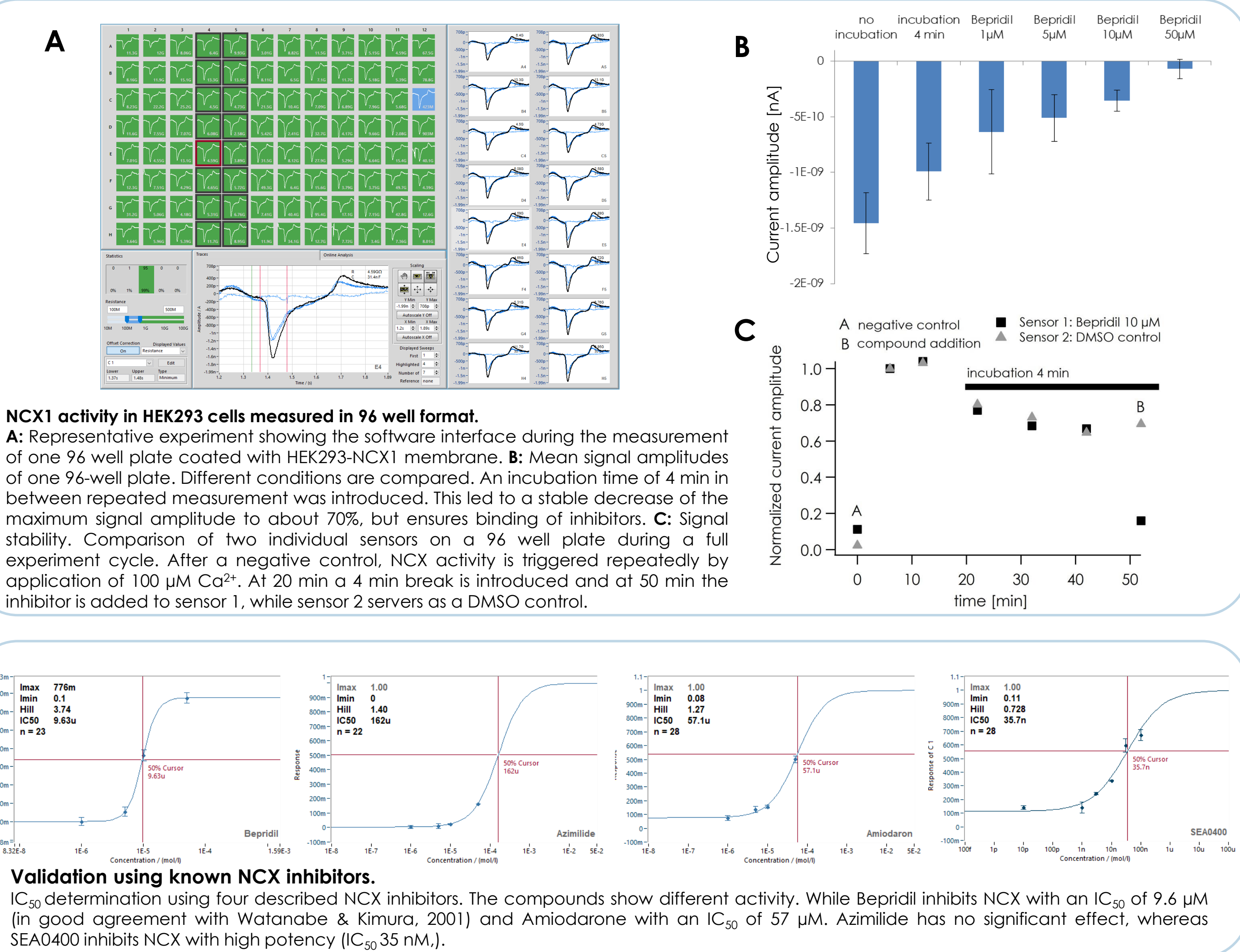
Calcium Affinity
iCell: $\text{KM} = 125 \mu\text{M}$
HEK NCX1: $\text{KM} = 74 \mu\text{M}$

Direct measurement of NCX activity with SSM-based electrophysiology.
A: After a rinse with a sodium containing buffer cardiac NCX was activated with $300 \mu\text{M}$ calcium. **B:** A transient negative current indicates a net outward current. **C:** Calcium affinity of NCX1 recorded from HEK cells ($74 \mu\text{M}$) was comparable with iCell Cardiomyocytes ($125 \mu\text{M}$). **D:** NCX1 in HEK cells, iCell Cardiomyocytes² and Cor.4U cells was blocked by KB-R7943 in a concentration-dependent manner with similar IC_{50} values. The IC_{50} values agreed well with the literature (Matsuda et al., 2001). **E:** SEA0400 is a specific blocker of NCX (Matsuda et al., 2001). SEA0400 blocked NCX activated by $300 \mu\text{M}$ Ca^{2+} with IC_{50} in good agreement with the literature (Matsuda et al., 2001).



SEA0400 inhibition increases the beat rate of iPSC derived CMs. iCell Cardiomyocytes² were seeded on the CardioExcyte96 well plate and the beat rate was determined via impedance recordings. Addition of SEA0400 significantly increased the beat rate in a dose dependent manner. **A:** Individual traces comparing the development of the beat rate in one well shortly before and 4h after addition of SEA0400. **B:** Time-dependent, long-term effect of SEA0400 on the beat rate of iCell Cardiomyocytes² (average of all wells, SD).

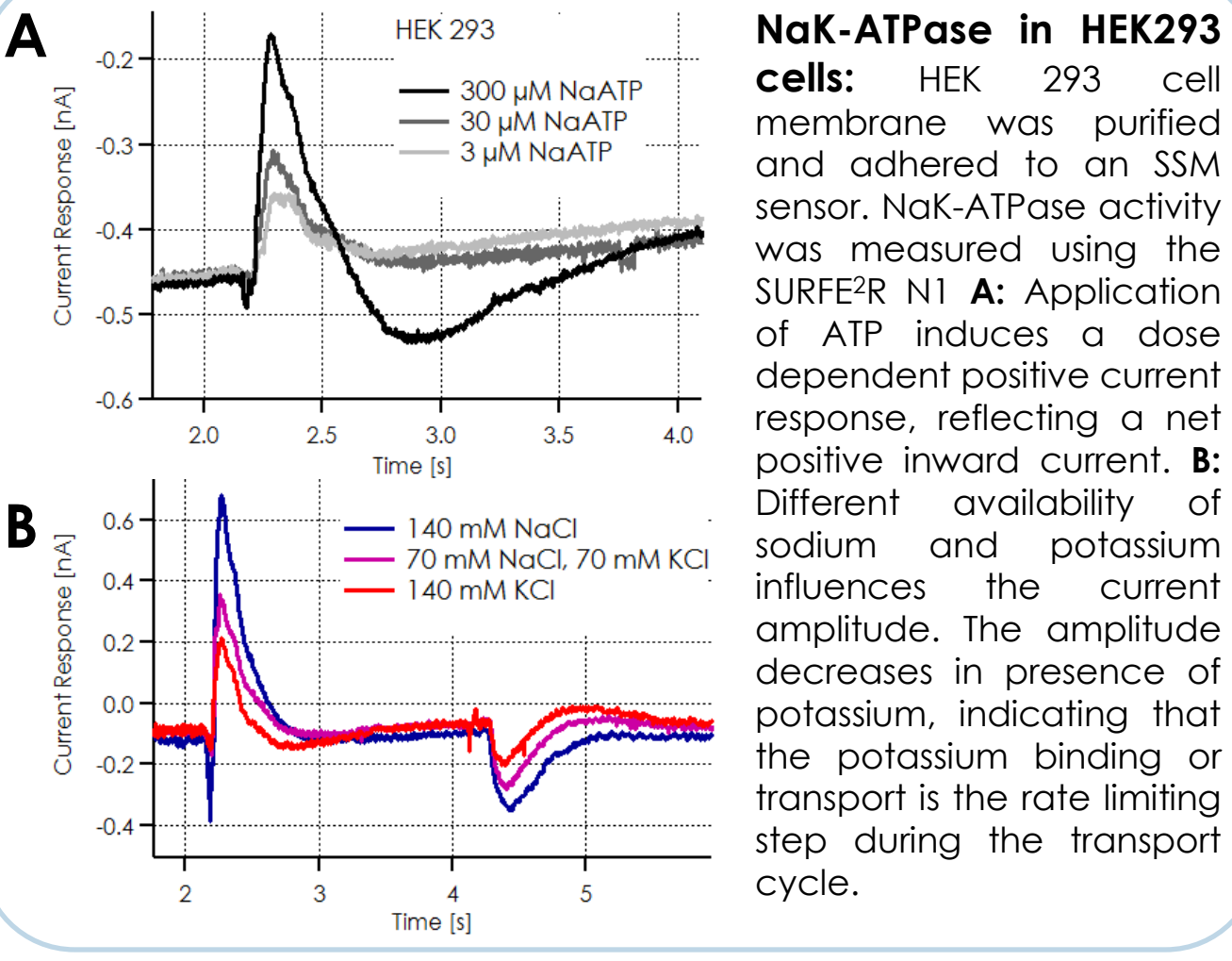
Development of a screening tool for NCX



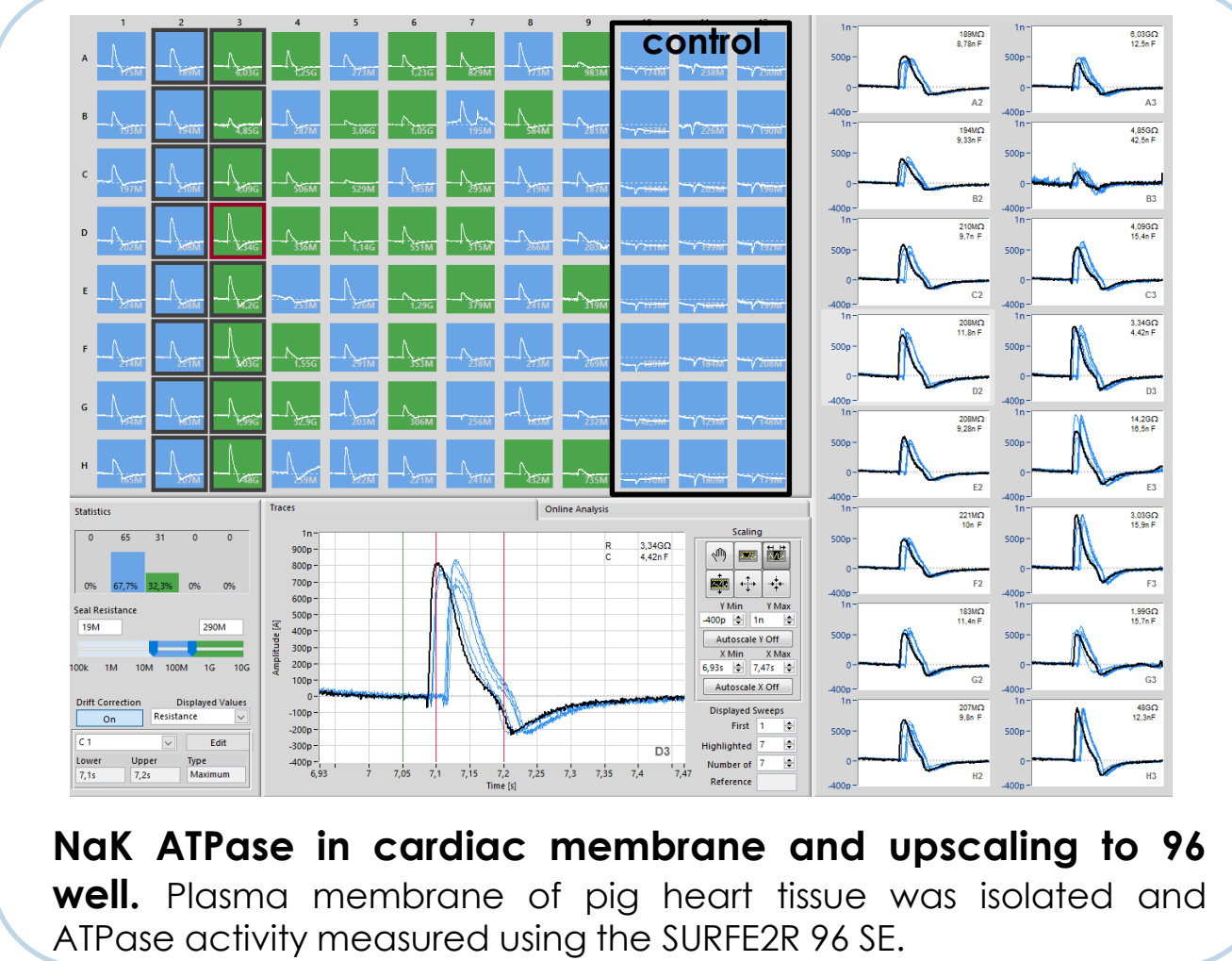
NCX1 activity in HEK293 cells measured in 96 well format. **A:** Representative experiment showing the software interface during the measurement of one 96 well plate, coated with HEK293-NCX1 membrane. **B:** Mean signal amplitudes of one 96-well plate. Different conditions are compared. An incubation time of 4 min in between repeated measurement was introduced. This led to a stable decrease of the maximum signal amplitude to about 70%, but ensures binding of inhibitors. **C:** Signal stability. Comparison of two individual sensors on a 96 well plate during a full experiment cycle. After a negative control, NCX activity is triggered repeatedly by application of $100 \mu\text{M}$ Ca^{2+} . At 20 min a 4 min break is introduced and at 50 min the inhibitor is added to sensor 1, while sensor 2 serves as a DMSO control.

Validation using known NCX inhibitors. IC_{50} determination using four described NCX inhibitors. The compounds show different activity. While Bepidil inhibits NCX with an IC_{50} of $9.6 \mu\text{M}$ (in good agreement with Watanabe & Kimura, 2001) and Amlodarone with an IC_{50} of $57 \mu\text{M}$. Azimilde has no significant effect, whereas SEA0400 inhibits NCX with high potency (IC_{50} 35 nM.).

NaK-ATPase in different cells



NaK-ATPase in HEK293 cells: HEK 293 cell membrane was purified and adhered to an SSM sensor. NaK-ATPase activity was measured using the SURFER N1. **A:** Application of ATP induces a dose dependent positive current response, reflecting a net positive inward current. **B:** Different availability of sodium and potassium influences the current amplitude. The amplitude decreases in presence of potassium, indicating that the potassium binding or transport is the rate limiting step during the transport cycle.



NaK ATPase in cardiac membrane and upscaling to 96 well. Plasma membrane of pig heart tissue was isolated and ATPase activity measured using the SURFER 96 SE.