Materials and Methods

Isoproterenol (Catecholamine) Induced Cardiomyopathy: Isoproterenol (ISO) induced cardiomyopathy was produced in cats at 5-6 months of age. These animals were divided into three groups and sacrificed at Day 10 (injury), Day 17 (early recovery), or Day 38 (late recovery) following ISO-filled minipump implantation[1]. Cardiomyopathy was induced by a continuous infusion of L-isoproterenol HCl dissolved in isotonic saline at a rate of 1100 µg/kg/hr from two Alzet minipumps (model #2ML1). Minipumps were implanted subcutaneously via a small incision in the dorsal region of the clavotrapezius and acromiotrapezius muscle, under general anesthesia. The animals were sedated with ketamine (50 mg/kg) and acepromazine (0.1 mg/kg) IM for induction of anesthesia, and then maintained on 1-2% isoflurane (nasal cone) in 100% oxygen throughout the procedure. The area within the incision was injected with Bupivacaine (0.5 ml) to reduce pain, and the skin of the animals was sutured and closed with metal staples. Animals were given meloxicam (0.3mg/kg SC, IM) and enrofloxacin (5mg/kg IM) following surgery. The rate of infusion from the minipump was 10 µl/hr, which delivers 1100 µg/kg/hr of isoproterenol for a period of 7 days. This dose (1100 ug/kg/hr) was determined in a preliminary dosing study. Ten days following pump implantation the pumps were removed. Respiratory distress (severe labored breathing) and/or pain were monitored rigorously. If any of these aforementioned symptoms arose the animal was treated with a diuretic for pulmonary edema and/or meloxicam for pain. During a pilot study (data not shown) we observed several cases of pulmonary edema that were successfully treated with one to two treatments with a diuretic, and we did not observe any signs of pain or distress after treatment with pain medication following surgical procedures.

Echocardiography: After thoracic shaving, ECHO measurements of atrial and ventricular chamber dimensions and cardiac function were made. Echocardiographic (ECHO) evaluation of cardiac structure and function was performed for baseline assessment within one week prior to minipump insertion[2]. With baseline information we were able to monitor each animal's cardiac function following implantation of the ISO filled mini-osmotic pumps. ECHO was performed for follow-up assessment at 7, 10, 17 and 38 days following implantation of ISO minipumps. The animals were sedated with ketamine (50 mg/kg) and acepromazine (0.1 mg/kg) IM. Serum was separated from blood withdrawn from the basilic vein by venipuncture prior to echocardiography and stored for analysis of circulating cardiac Troponin I levels. These measurements were critical for determining each animal's cardiac function during injury and recovery protocols.

BrdU Labeling: 5-bromo-2'-deoxyuridine (BrdU) is a thymidine analog that incorporates into DNA upon replication and repair, making it a potential marker for dividing cells. Osmotic minipumps (ALZET) containing the DNA labeling reagent 5'-bromo-2'-deoxyuridine (BrdU) at a concentration of 50 mg/ml, dissolved in 50% de-ionized water/50% DMSO, were implanted subcutaneously[3]. The use of ALZET osmotic pumps allows a constant infusion of the marker for the length of time required to ensure incorporation of BrdU in the slowly dividing tissue of the myocardium. The procedure was performed one week prior to euthanasia for control and ISO treated animals. It was also utilized in a pulse-chase manner, in a small group of animals, by implanting BrdU minipumps during the ISO injury phase, removing all minipumps at Day 10, and then waiting until Day 38 for euthanasia and explant of the heart, to track the fate of BrdU labeled cells over a longer period of time. The surgical procedures used for BrdU minipump implantation were the same as those for ISO minipump implantation.

Heart Extraction and Perfusion Fixation: In preparation for euthanasia, animals were sedated with ketamine (50 mg/kg) and acepromazine (0.1 mg/kg) IM and then received an intraperitoneal injection of sodium pentobarbital 50 mg/kg. To insure that the animal was at the

appropriate depth of anesthesia, withdrawal reflex, absence of response to toe pinch, and eye reflex were tested. A cardiectomy was then performed. A cannula was inserted into the aorta and secured. A small saline filled balloon was inserted through the LA into the LV and filled to and kept at a diastolic pressure of 6 mmHg. The heart was arrested in diastole with infusion of cadmium chloride and perfusion fixed for 45 minutes with 10% buffered formalin. The heart was then kept submerged in 10% formalin at 4°C for 48-72 hours before tissue processing[3].

Tissue Section Histochemistry: Formalin fixed hearts were divided into left and right ventricular, apex, middle, and base components. The left and right atria were also separated. The tissue was processed, embedded in paraffin and cut as 5 μm sections. Histochemistry included Masson's Trichrome stain for determination of replacement fibrosis and overall tissue damage. Masson's Trichrome stained slides were quantified using a microscope (Nikon) interfaced with an analog camera and a bioquantification software system (Bioquant Osteo II, Nashville, TN). The assessment and analysis of the data was carried out in a blinded fashion. Prior to acquisition, the camera was white-balanced to ensure that uniform background color was maintained. The microscope's light intensity was maintained at a constant level to ensure the background values were similar for each acquired image. Likewise, the f-stop for the camera was maintained at a constant level for each acquired image. Antibodies used for immunostaining: Laminin (Sigma), alpha-actin (Sigma), BrdU (Roche), cKit (Dako), CD45 (Novus), DAPI. Tyramide Signal Amplification (TSA^{TM} PerkinElmer USA) was used to amplify the signal for cKit and CD45.

Electrophysiology, Contractions, and ISO Response: These techniques have been described in detail in previous publications[1, 4-10]. Myocytes were isolated by perfusion of the heart with a Ca^{2+} free solution containing collagenase. When the heart tissue softened, tissue was minced, washed in normal physiological solutions containing 1 mM Ca^{2+} and kept at room temperature. Rod shaped, quiescent myocytes were used for electrophysiological and contractile studies within 12 hours of isolation.

For contraction studies, myocytes were placed in a chamber mounted on the stage of an inverted microscope and perfused with Tyrode's solution at 35^{o} C. Myocytes were loaded with the Ca^{2+} indicator fluo-4(AM), washed and then field stimulated to induce contractions and Ca^{2+} transients.

L-type Ca^{2+} current was measured with established techniques. Myocytes were placed in a bath mounted on the stage of an inverted microscope. Membrane current and voltage were determined with standard single electrode voltage clamp techniques. Pipette and bath solutions were Na free, to eliminate Na and Na/Ca^{2+} exchanger currents. Bath Ca^{2+} was 2 mM. Membrane potential was held at -90 mV and test steps were in 10 mV increments, up to +60 mV.

References

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Supplemental Figure Legends

Supplemental Figure I. ISO induced injury model protocol: A: Isoproterenol (ISO) was continuously infused via an osmotic minipump for 10 days to induce cardiomyopathy. BrdU was infused by osmotic minipump for 7 days at the times shown. One group of animals was euthanized at Day 10. A second group of animals had BrdU minipumps inserted at Day 10 and were euthanized at Day 17. A third group of animals had BrdU minipumps inserted on Day 31 and were euthanized on Day 38. **B:** A forth set of animals underwent a pulse-chase protocol, with ISO-induced injury in the usual fashion, and BrdU minipumps during the injury phase (Pulse). However, both sets of minipumps were removed at Day 10 (Pulse) and animals were euthanized at Day 38 (Chase).

Supplemental Figure II. Serum analysis: Cardiac injury was assessed with serum analysis of blood samples drawn from the basilic vein. Serum was analyzed for the cardiac injury biomarker, Troponin I, by Antech Diagnostics (Morrisville, NC). A significant increase in Troponin I levels was found at Day 3 and this returned to normal levels by Day 10. Troponin I values below the detection limit were ascribed the lowest detectable value of the assay (0.2 ng/ml) for statistical tests. Baseline and Day 3 (N=6); Day 10 (N=2); Day 17 (N=3); Day 38 (N=4). *P<0.05; **P<0.01; ***P<0.001 versus Baseline. Data are presented as mean ± SEM.

Supplemental Figure III. ECHO derived changes in left ventricular wall thickness: Wall thickness increased following ISO-induced injury. Isoproterenol (1100 ug/kg/hr) caused ventricular dilation but wall thickening was also observed, documenting hypertrophy. Baseline through Day 10 (N=19); Day 17 (N=12); Day 38 (N=6). *P<0.05; **P<0.01; ***P<0.001 versus Baseline. Data are presented as mean ± SEM.

Supplemental Figure IV. Masson's Trichrome staining of cardiac tissue from ISO injured feline left ventricle (Day 10). A representative focal area with more extensive damage is shown. Collagen (Blue); cardiac tissue (Red).

Supplemental Figure V. A/B: Show the effects of 1 mM ISO on the Ca^{2+} current in control (**A**) and ISO injured (**B**) myocytes. **C-H:** Shows the effects of 1 mM ISO on contractions of control (**C/E**) and ISO injured (**D/E**) and Ca^{2+} transients of control (**F/H**) and ISO injured (**G/H**) ventricular myocytes. (**I**) Exposure of LV myocytes to ISO caused an increase in the L-type Ca^{2+} current in control myocytes, but had very small effects on myocytes from ISO injured hearts (**J**).

Supplemental Figure VI. Additional examples of BrdU+ myocyte and non-myocyte nuclei are shown. A merged image is shown (**A**), and α-Actin (**B**), BrdU (**C**) and DAPI (**D**) are shown for the same section. Non-myocyte and myocyte nuclei are easily identified.

Supplemental Figure VII: Representative examples of "bright" and "dimly" labeled BrdU+ myocyte and non-myocyte nuclei are shown in a left ventricular section from a pulse-chase experiment, with BrdU minipumps removed at Day 10 and animals sacrificed at Day 38. DAPI (**A**), Laminin (**B**), α-Cardiac Actin (**C**), BrdU, (**D**), and a merged image (**E**) are shown for the same section. BrdU+ non-myocyte and myocyte nuclei are easily identified. The arrows in **D** and **E** point to one "bright" and four "dimly" labeled BrdU+ cardiac myocyte nuclei. Bright and dimly BrdU+ nonmyocytes are also shown.

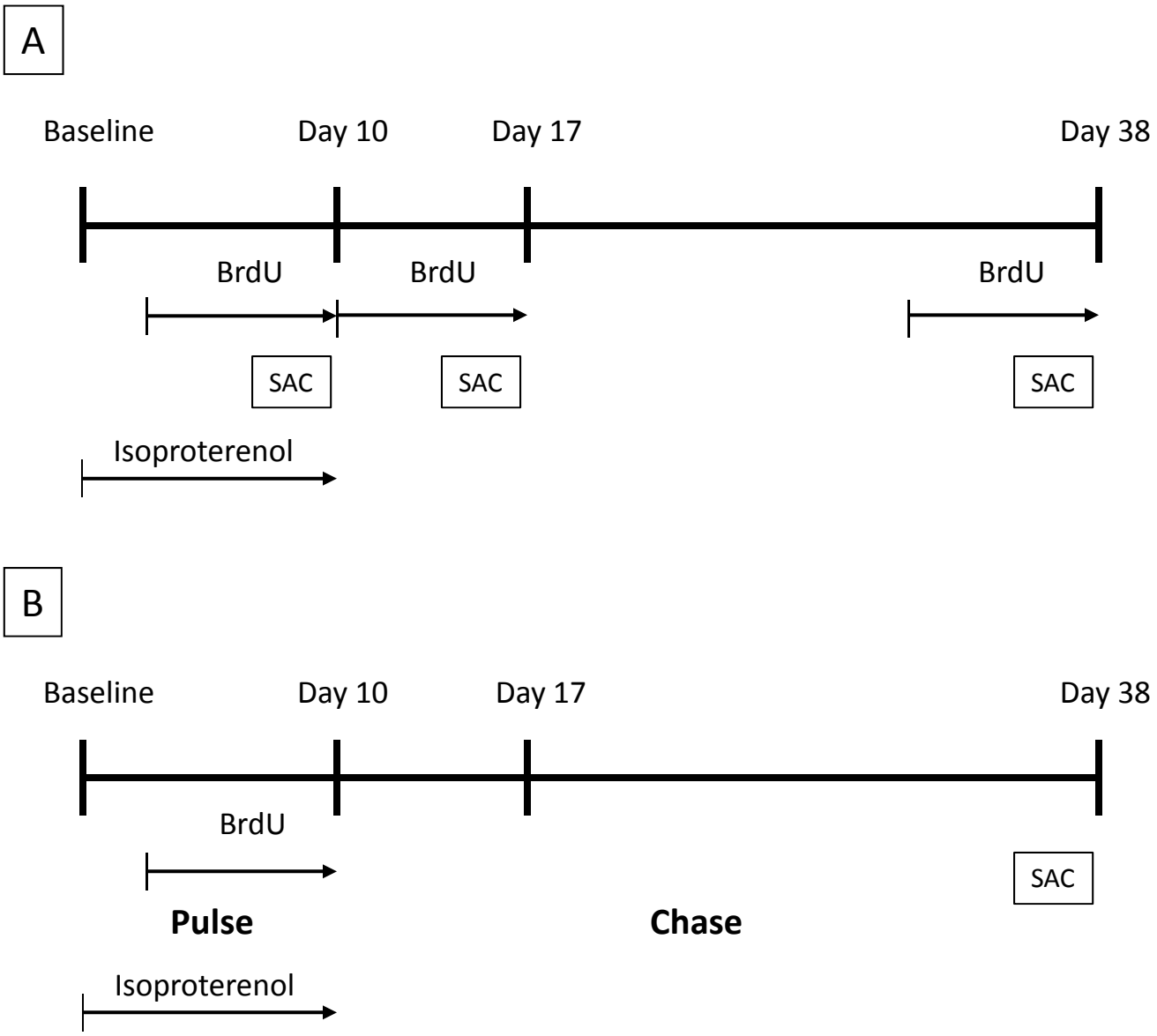
Supplemental Figure VIII. Examples of cKit and BrdU staining in control ventricular tissue (**A**) and in ISO injured ventricular tissue at Day 10 (**B**). **C-F:** Additional examples of cKit+ cells at Day 10. cKit (Red); BrdU (Green); DAPI (Blue).

Supplemental Figure IX. CD45+ cells in the heart proliferate during ISO induced injury: A: In the LV CD45+ cells proliferate only during ISO injury. **B:** The % of CD45+ cells in the left ventricle did not change during the time course of the study. **C/D:** Similar results were observed in the atria.

Supplemental Figure X. Representative examples of CD45+ cells in the ventricle are shown in control tissue (**A-C**) and in the ISO-injured ventricle at Day 10 (**D-K**). Control (**A-C**) and Day 10 (**D-K**)**:** CD45 (Green); BrdU (Red); Nuclei (Blue).

Supplemental Figure XI. Cartoon depicting our interpretation of our composite results with the timing and source of new myocytes after ISO injury: A: In animals with ISO injury (Days 0-10) and BrdU infusion during Days 10-17, we observed BrdU+ new myocytes at Day 17. Our results suggest that these myocytes are derived from cKit+ cardiac precursors that were activated during the injury phase and then commit to the cardiac lineage (without proliferating). We suggest that BrdU incorporates into these new myocytes during a brief period of new myocyte proliferation after removal of ISO. **B:** In animals with BrdU infused during ISO injury and studied at Day 10 we found BrdU labeled cKit cells, but no increases in new myocytes. When these animals were sacrificed at Day 38 (pulse-chase) we observed a reduced % of BrdU labeled cKit cells and an increase in BrdU labeled myocytes. These results suggest that cKit cells proliferate during injury but do not commit to the myocyte lineage until ISO pumps are removed at Day 10. After removal of ISO the “activated” cKit cells differentiate into new myocytes (without proliferating) and then these new myocytes have a brief proliferative phase before exiting the cell cycle. Red cell membranes depict cKit cells. Burgundy nuclei are BrdU- and yellow nuclei are BrdU+. Striated cells are myocytes.

Isoproterenol Induced Cardiac Damage



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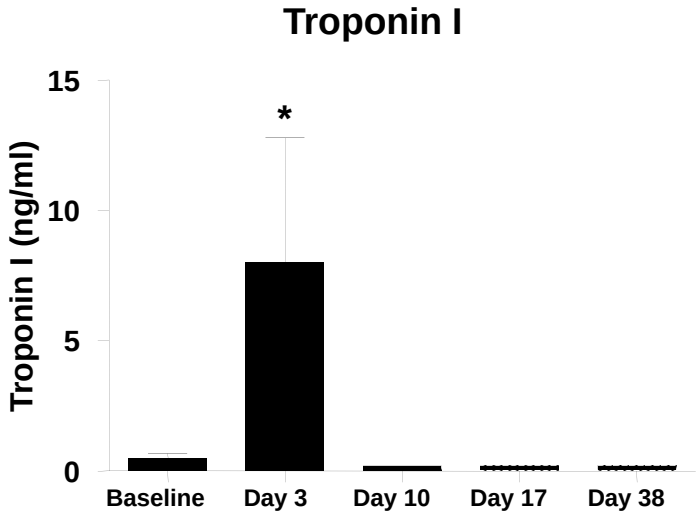
Troponin I: Serum Analysis



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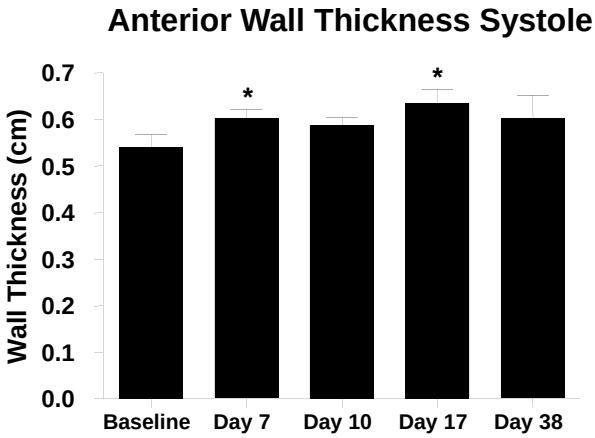

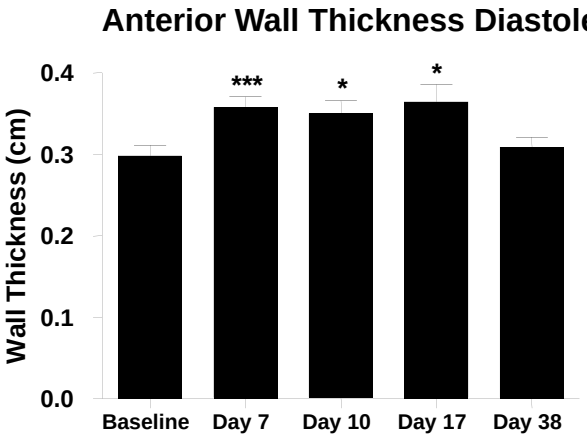

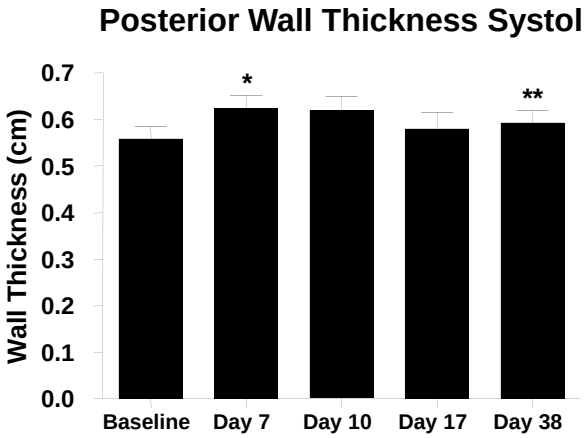

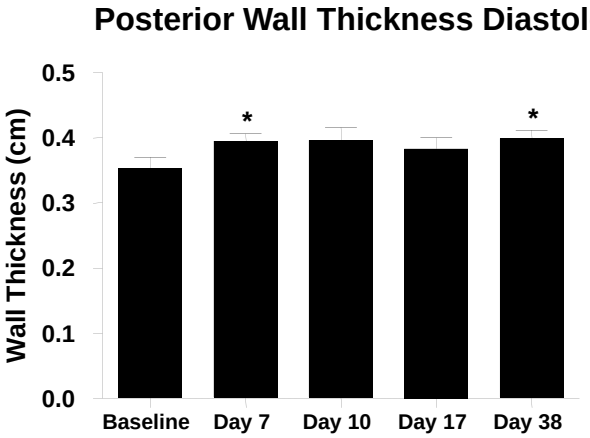

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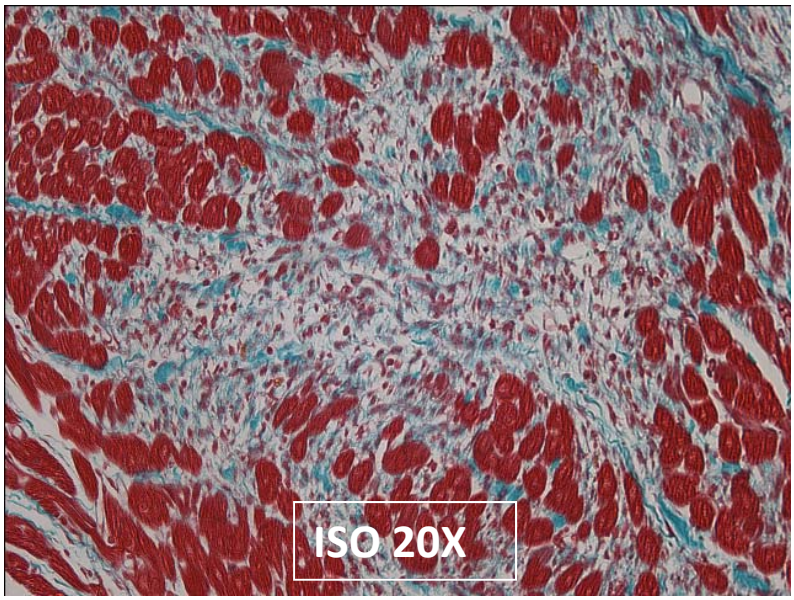

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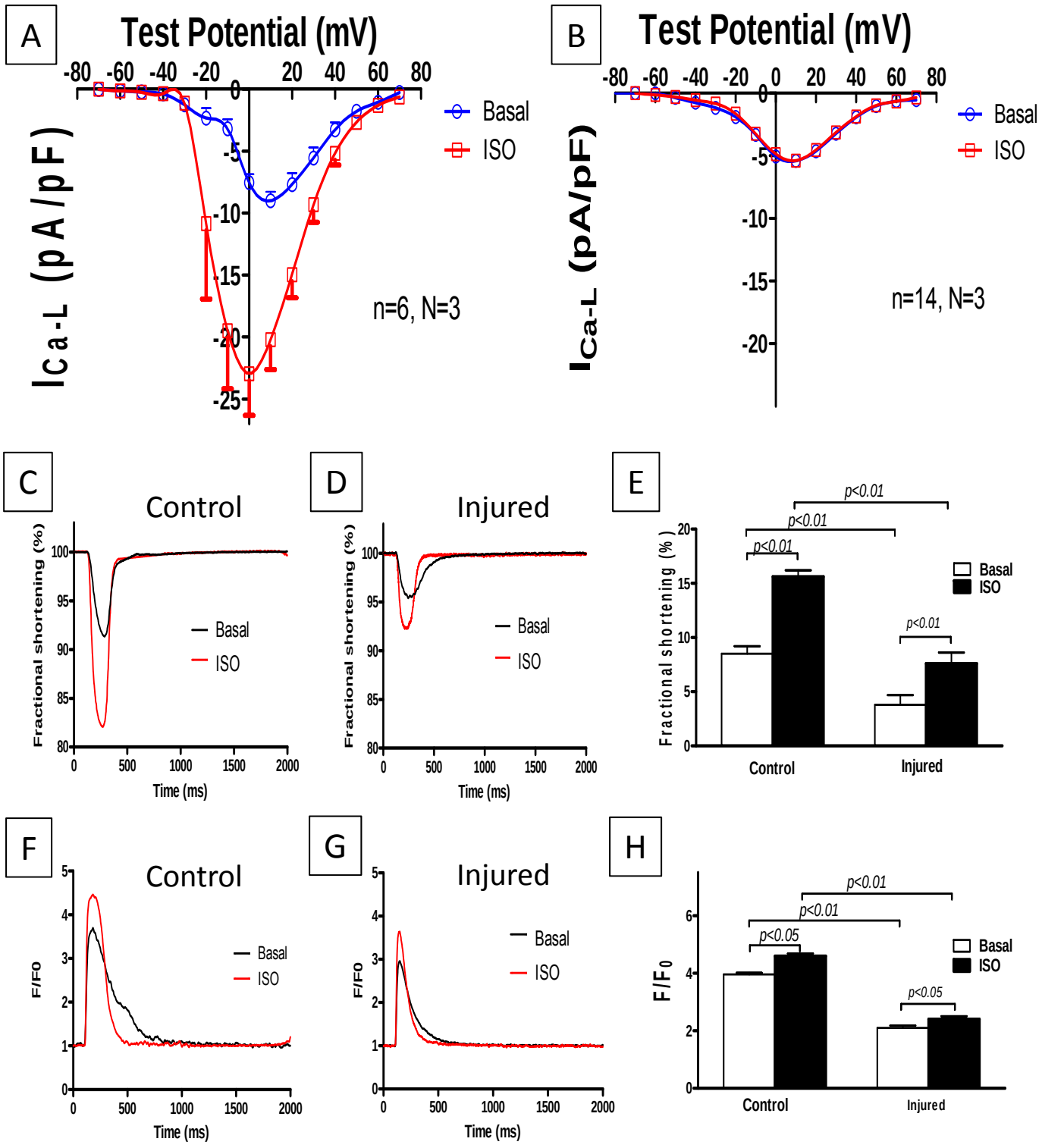

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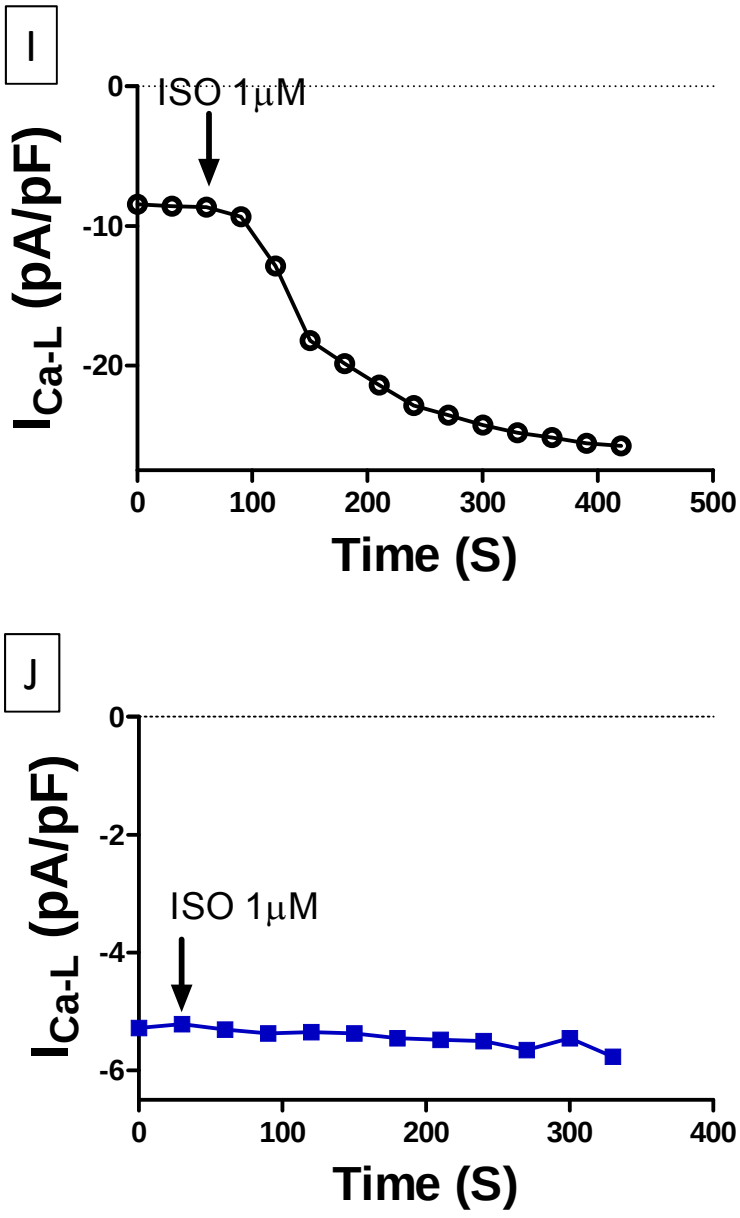

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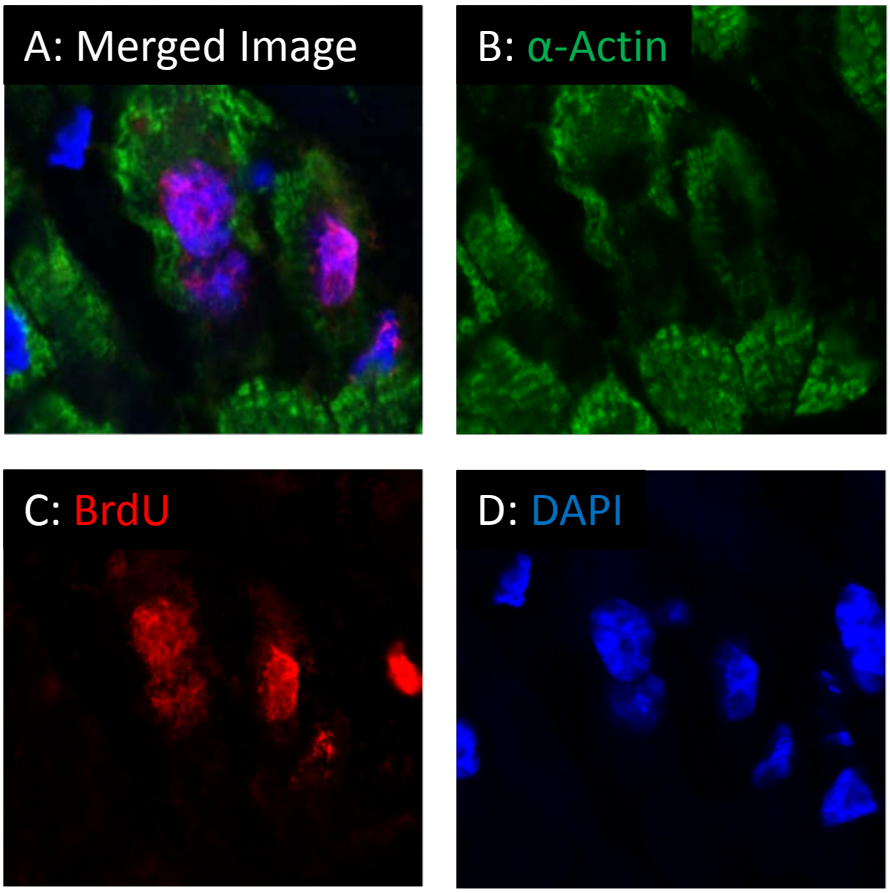

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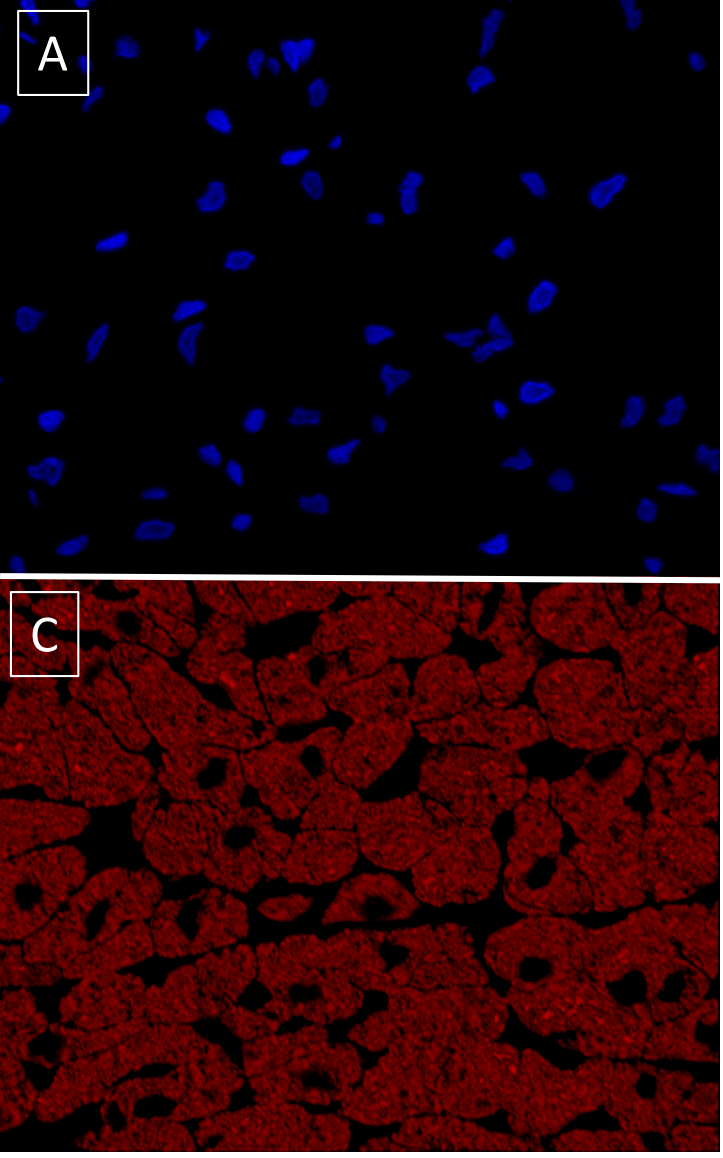

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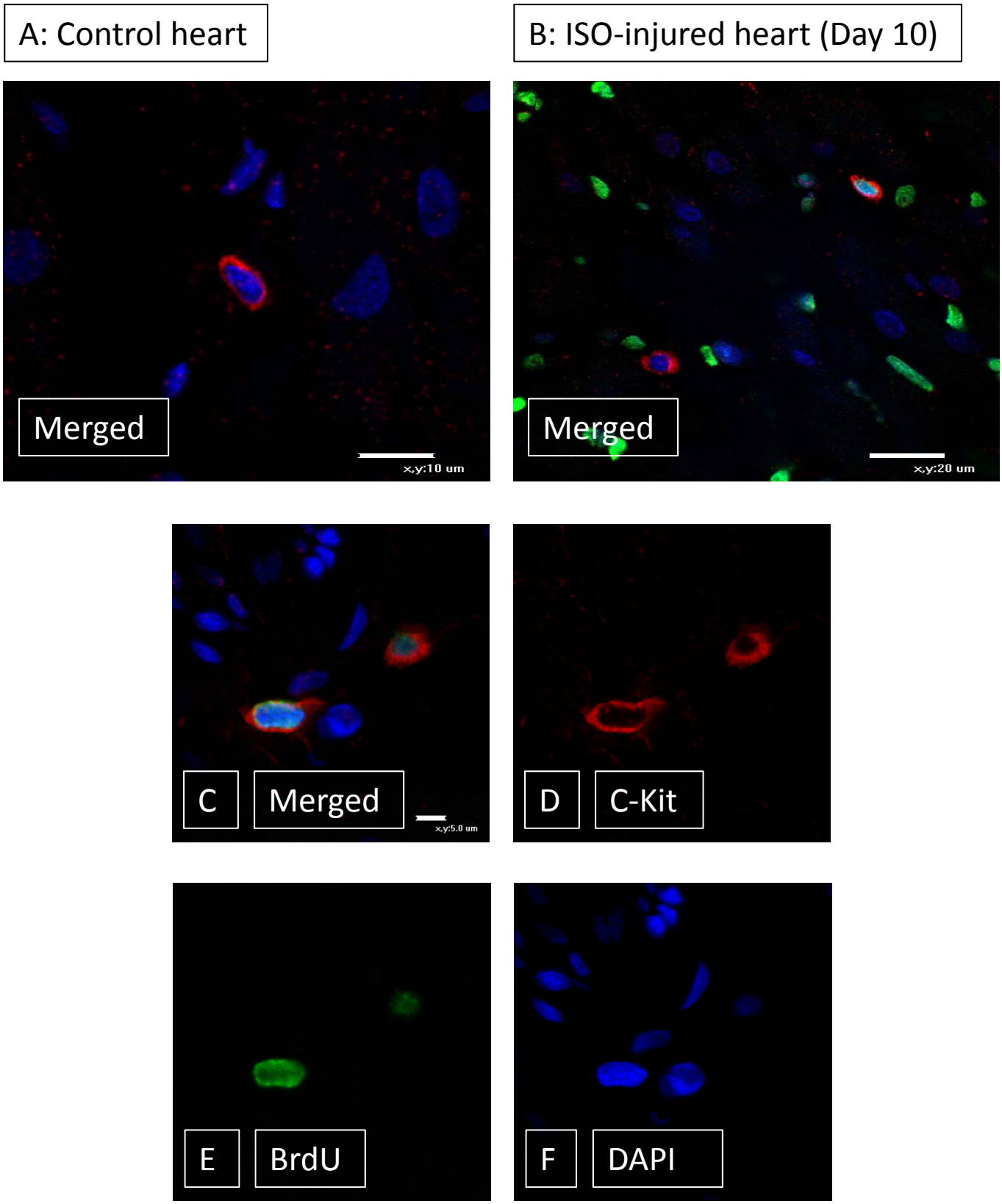

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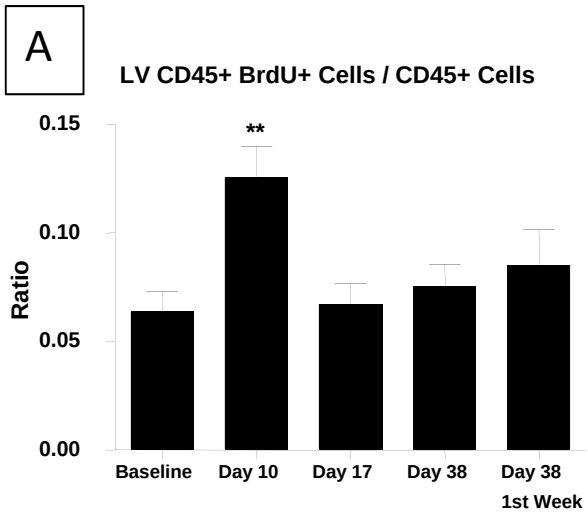

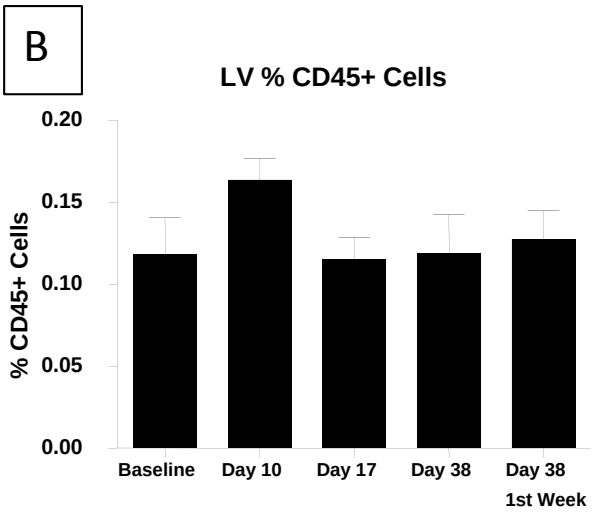

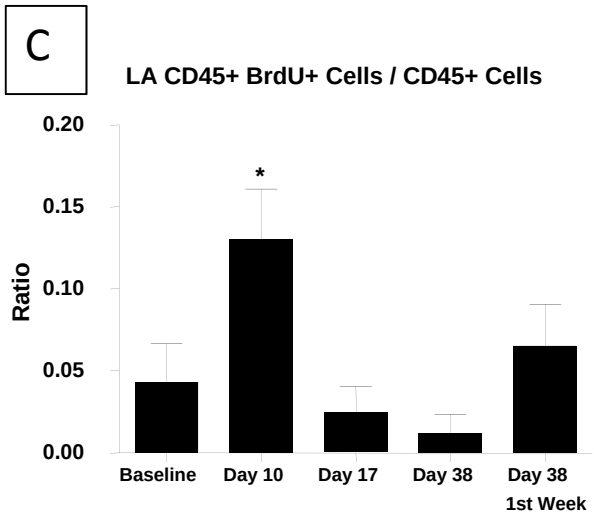

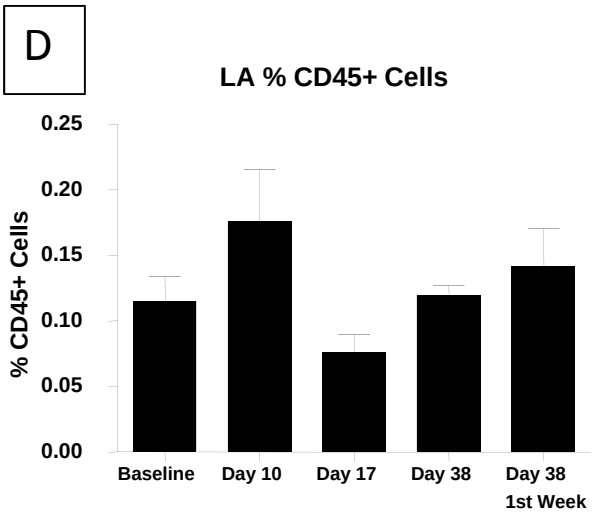

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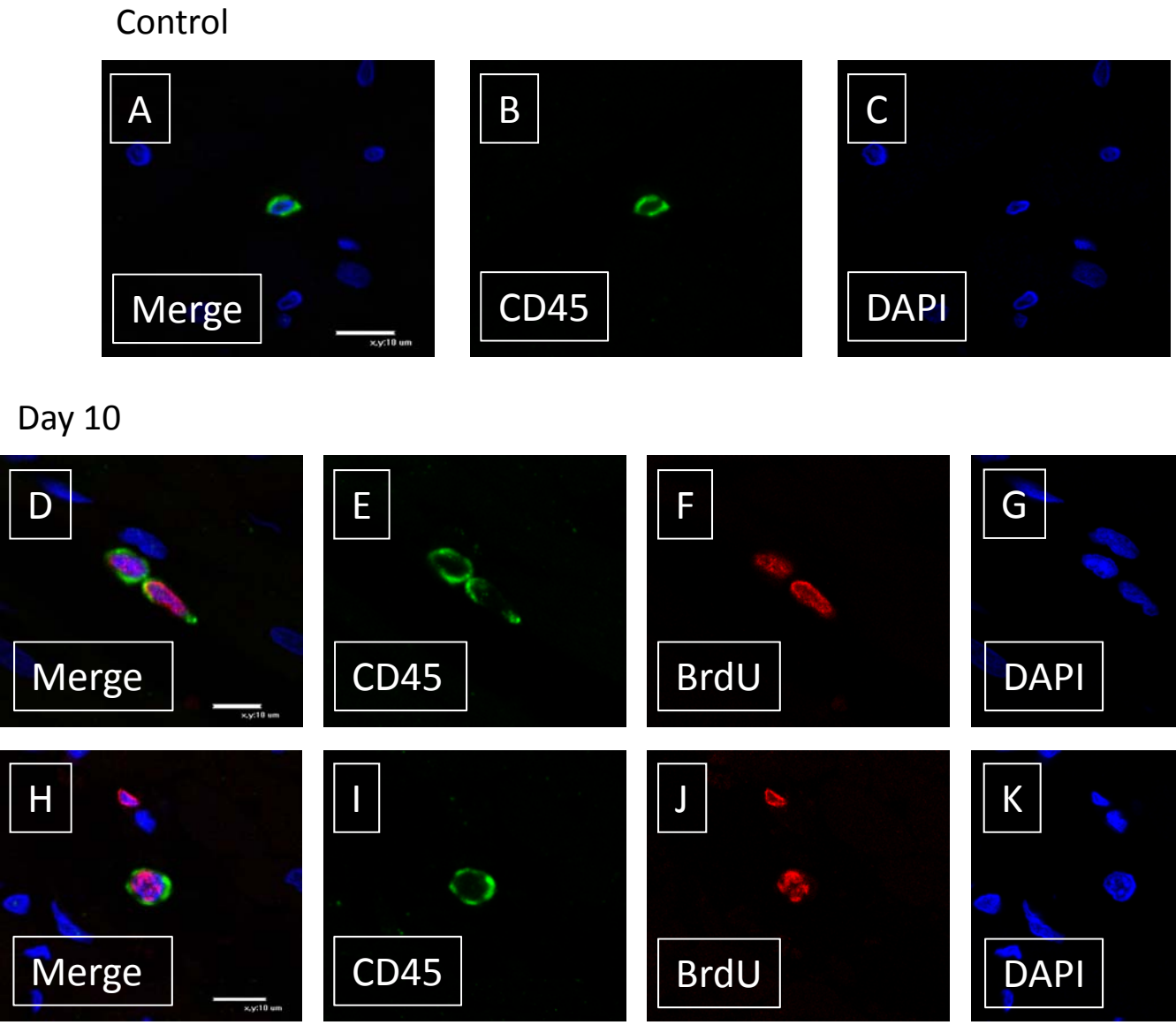

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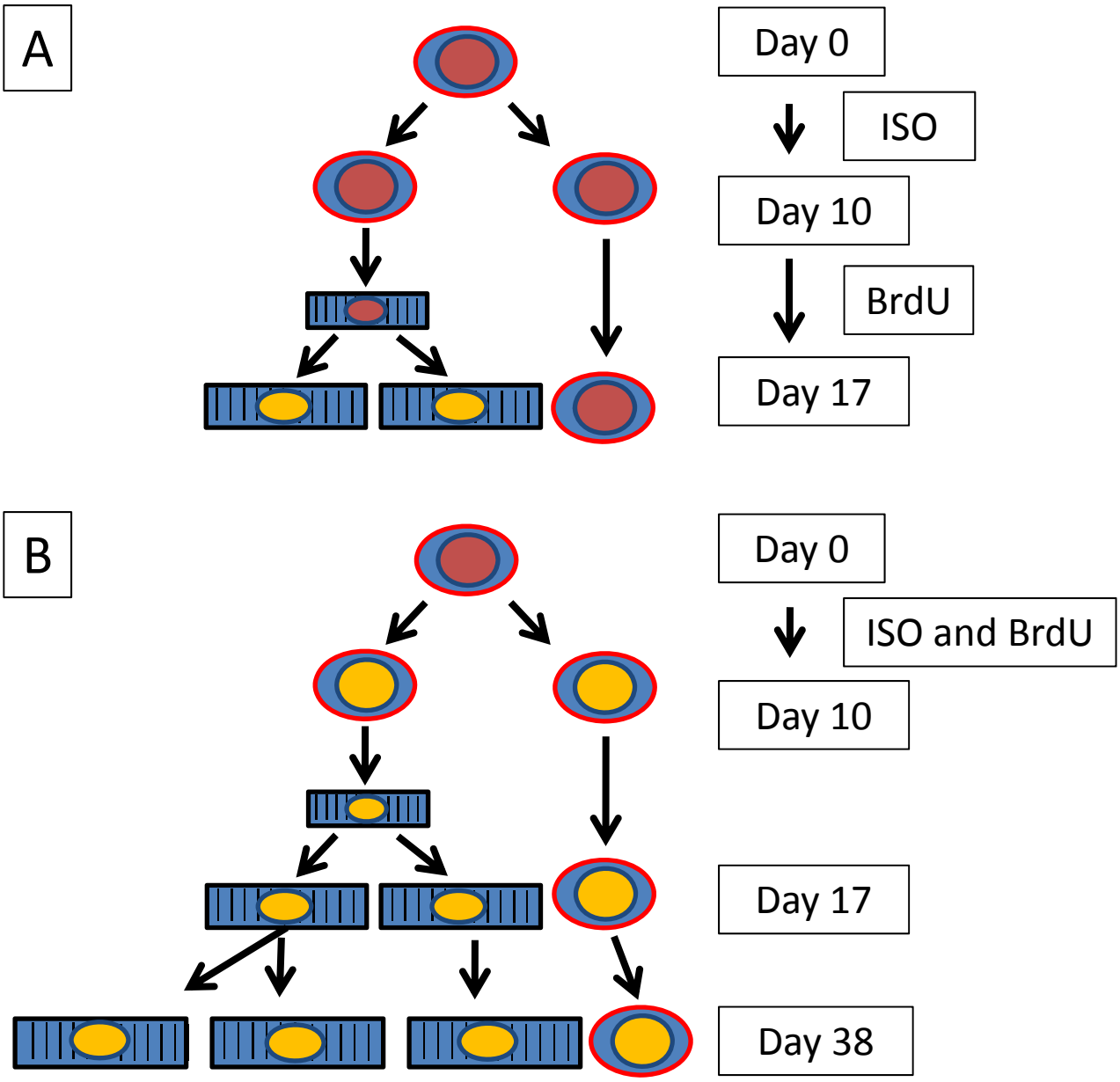

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