A flexible Teensy microcontroller based interface for image acquisition and behavioral monitoring across diverse behavioral neuroscience platforms

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**Abstract**

Systems neuroscience research requires the integration of precisely timed data acquisition with measures of behavioral output. While many commercial systems have been designed to meet these needs, they are prohibitively expensive to use and fail to offer flexibility that allows for integration across diverse experimental designs. We here describe a Teensy 3.2 microcontroller-based interface that offers fast and precisely timed digital signal readout for data acquisition with both digital and analog output to control behavioral experiments. We demonstrate the efficacy and temporal precision of the Teensy based interface in two experimental settings with different demands: (1) reliable, high-accuracy capture of directional movement on a spherical treadmill combined with high speed camera control for monitoring neurons in real-time and (2) a behavioral learning paradigm that delivers auditory and visual cues to task performing animals while monitoring activity through integrated camera control. Both setups provide accuracy that rivals commercially available designs. We conclude that the Teensy 3.2, in conjunction with Teensy hardware modules, provides an optimal form of experimental control, particularly for those interested in the integration of imaging with behavior output in laboratory experiments.

**Introduction**

High-speed imaging in behavioral neuroscience research is a technique that demands high temporal fidelity and precise integration between the imaging platform and animal behavior. The recent use of calcium imaging to track the activity of individual cells at millisecond resolution while animals engage in behavior represents such a requirement (Klaus, et al., 2017; Barbera, et al., 2016; Mohammed, et al., 2016; Markowitz, et al., 2018). Strict alignment of neuronal signals with experimental inputs or outputs at these sampling frequencies is essential (Solari, Sviatkó, Laszlovsky, Hegedüs, & Hangya, 2018). This importance of this precision will become even more relevant with the emergence of faster calcium indicators or new voltage imaging sensors that utilize sampling rates up to 1 kHz (Yoav, et al., 2018). A new challenge is to find flexible, intuitive, and accurate ways to integrate experimental control, behavioral output, and data sampling in a way that is synchronized and easy to use.

Experiments that examine the neural basis of behavior typically require precisely timed data acquisition and command signals, as noted previously for electrophysiology recordings (Solari, Sviatkó, Laszlovsky, Hegedüs, & Hangya, 2018). This type of experimental control has traditionally involved the use of expensive lab laboratory equipment for the control and precision timing of stimuli. These often involve producing a script using commercial software (i.e., Labview, MATLAB) that triggers the cameras to record while providing experimental control through executed through an expensive Analog to Digital Data Acquisition interface (i.e., National Instruments) running on a personal computer using the associated drivers. This complex system may also require additional stimulus generators to produce the analog or digital signals needed to perform this experiment. Most importantly, while highly precise, the use of such equipment is prohibitively expensive to users outside of well-funded research or industry laboratories.

Over the last several years, the emergence of small highly precise microcontrollers for use by hobbyists have gained traction across a variety of scientific fields (cite) .These microcontrollers aresmall, affordable, open-source and while still precise, allow for customization and easy implementation with a low initial cost. Such a system would allow many individuals to explore scientific questions with low barriers to entry. Two such microcontrollers (the Teensy 3.2 or Arduino UNO) are capable of delivering precisely timed pulses with microsecond-level resolution using user-friendly functions, which can be used to coordinate experiments. While more expensive equipment is commonly used for data acquisition and experimental control, these microcontrollers are far less expensive, are sufficient for the majority of experimental needs, and leverage open-source software and intuitive programming languages (D'Ausilio, 2012). The Arduino programming environment, which the Teensy utilizes, is simple to learn for anyone with any programming background, and utilizing this type of microcontroller doesn’t require much experience in the realm of electronics, as explained in depth previously (D'Ausilio, 2012). In addition to the standard features that the Arduino UNO, for example, the Teensy 3.2 also delivers true analog output, which the Arduino UNO lacks. An open source Audio library available only for the Teensy, , adds more functionality by providing a simple way to create and/or play sounds directly from the device. Therefore, operant conditioning experiments that utilize sound do nott necessitate additional audio equipment or knowledge of electrical circuits, aside from an inexpensive direct plug-in amplifier for the microcontroller and a speaker.

Camera control via an Arduino device that initiates only the start of an imaging sequence has been previously shown (Micallef, Takahashi, Larkum, & Palmer, 2017). However, Arduino and Teensy devices can instead be used to precisely time imaging capture for each frame. A common technique in laboratory studies using more expensive AD converters is to set up an imaging device to utilize an “external trigger”, where the rising phase of a digital pulse or TTL pulse either initiates a sequence of internally clocked image captures. One possibly concern with this approach is that imprecise triggering of each frame based on a different digital pulses could introducejitter in digital pulse delivery causing frame loss and can also necessitate interpolation for many statistical analyses. In particular, behavioral data must be precisely aligned to imaging data in experiments that utilize imaging. Thus, there currently exists a need to engineer a device capable of delivering continuous, precisely timed digital pulses that can synchronize other experimental events with camera control. The

Here, we demonstrate in two simple experimental paradigms that the Teensy 3.2 is indeed a simple, low-cost, flexible device capable of coordinating highly accurate data acquisition, and sound and stimulus delivery with image capture. In order to align experimental data with imaging data, frame capture should be instantiated on a frame-by-frame basis. The Teensy 3.2 is capable of keeping highly accurate and low-bias timing that allow it to reliably instantiate frame capture with highly regular intervals while delivering stimuli or recording experimental data with microsecond-level precision. This removes the need for post-hoc interpolation or time-alignment between different external devices.

**Methods**

In order to demonstrate the flexibility of the Teensy 3.2 in designing precisely timed imaging-based experiments, we designed two experimental setups: one that utilizes motion tracking on a floating Styrofoam ball while delivering regular digital pulses at a typical image-capturing frequency, and one that utilizes the Teensy’s Audio library in a trace conditioning paradigm while again delivering regular digital pulses.

*Motor acquisition experiment*

The overall design for this experiment is shown in Figure 1A. Two ADNS-9800 gaming sensor boards (https://www.tindie.com/products/jkicklighter/adns-9800-laser-motion-sensor/) were attached at the equator of a 3D-printed half-sphere in which a large, buoyant Styrofoam ball is floated by house air. These sensors lay at an angle of approximately 75 degrees from one another. This setup is adapted from that of Dombeck, Khabbaz, Collman, Adelman, & Tank (2007). The wiring of the two ADNS-9800 sensors to a Teensy 3.2 is demonstrated in Figure 2A.

To compute linear velocity, we used the y-readings of both sensors, and the rotational velocity can be computed using the x-readings. These two sensors were connected to a Teensy 3.2 via simple serial peripheral interface (SPI) connections with insulated 22 gauge wires , as shown in Figure 2A. The Teensy was connected to a PC using a USB-microUSB cable.

Due to the complexity of extracting motion data from these sensors, we utilized simple classes and functions that are freely available on Github (https://github.com/markbucklin/NavigationSensor) and abstract the complexity of acquiring motion data to a user-friendly level. In particular, this repository contains the ADNS9800 library, which is a modified version of the stock ADNS-9800 library (<https://github.com/mrjohnk/ADNS-9800>).

We modified the specific-use case of the library available in this repository to acquire data and send digital pulses every 50 milliseconds. In order to precisely time these events, we utilized the “IntervalTimer” function available in the standard Teensy library. This allows for microsecond-level precision in calling different functions using interrupts. Here, we used it to call a main function that sends out a digital pulse to trigger a frame capture, collects data from the two ADNS-9800 sensors, and sends the motion data to a main computer. The Teensy also has the very useful “ellapsedMicros” and “ellapsedMillis” libraries built in to the Teensyduino library, which, to microsecond or millisecond accuracy, respectively, act as time accumulators. These can also be used for precisely timing events as well. Though these can be downloaded separately for the Arduino, they come preinstalled in the Teensyduino library.

Via the ADNS9800 library, we read from the “motion burst” register of each sensor. On every call to the main function, we acquired the accumulated displacement over the previous 50 milliseconds in both the x and y directions. For the counts per inch setting we used a value of 3400 counts per inch, the default setting. As previously mentioned, during this interrupt, a digital “on” pulse that lasts for approximately 1 ms is sent out of a digital pin using the DigitalIO library (<https://github.com/greiman/DigitalIO>). This library allows us to use the functions “fastPinMode” and “fastDigitalWrite’, for example, which reduce the latency introduced by turning pins on, off, or setting their “mode” (to INPUT or OUTPUT, for example). Instead of using the default Arduino programming environment to upload our code to the Teensy, we used PlatformIO (<https://platformio.org/>), an add-on to the widely-used Atom text editor (<https://atom.io/>). This allowed us to easily build and upload our multi-folder library to the Teensy.

In order to begin experiments with the Teensy, after the main script was uploaded to the Teensy, we wrote a simple MATLAB-based graphical user interface that can be used on a desktop or laptop connected via a USB to the Teensy. In principle, however, this graphical user interface could be written in Python or any other programming language. Using this interface, the user enters the length of the experiment and the frequency of data acquisition. This frequency will determine the frequency with which digital pulses are sent to notify an external device such as a CMOS camera to capture an image, for example, and also determine the frequency with which accumulated motor information will be recorded by the PC. The PC or laptop sends this information over a serial connection to the Teensy utilizing a bidirectional microUSB-USB cable.

In a proof-of-concept experiment (Figure 3), we recorded a 10 minute long session of a mouse running on a 3 dimensional treadmill (Styrofoam ball floating on air), and data was acquired at 20 Hz concomitant with digital pulses that could be used to trigger a camera image capture or a different device. The mouse’s speed was computed using the y-coordinates of each ADNS-9800 sensor, and the total distance travelled at any one time point was computed using the following equation:

Where yR and yL are the y readings from the left and right sensors, and is the angle between the two sensors (75 degrees). Velocity was computed as the distance divided by the time between two adjacent frames. Times and distances travelled were recorded by the Teensy 3.2, and the timings of digital pulses were measured by an external device at 3051.76 Hz (Tucker-Davis Technologies RZ5D (TDT RZ5D)).

After analyzing the time stamps acquired by the TDT RZ5D system, we noticed that there was a very small timing drift (approximately 30 microseconds per second). To confirm that the frequency of data acquisition and timing of the corresponding digital pulses didn’t affect this drift, we repeated 5 minute recording sessions without a live mouse at 20, 50, and 100 Hz. These recordings used an identical script, except we embedded a 500 microsecond delay between the start and end of the digital pulse (“delayMicroseconds(500)”) instead of a 1 millisecond delay (“delay(1)”).

*Classical conditioning experiment*

To illustrate another simple experimental design wherein the Teensy 3.2 can be used to control four devices simultaneously while reliably outputting a sound, we created a trace conditioning experimental design. The general setup is shown in Figure 1B. In a trace conditioning experiment utilizing this setup, a head-fixed mouse would theoretically be exposed to a 9500 Hz tone concomitantly with a light stimulus. After, the mouse would receive a puff of air in its eyes. The goal is to train the mouse to blink upon exposure to the unconditioned stimuli.

In order to amplify the sound to a suitable volume, we added a “prop shield” to the Teensy. The prop shield is a very affordable, easy-to-use add-on that is capable of amplifying the analog output signal (shown in Figure 2B as pin A14). If stereo output were desired, the manufacturer also offers a true audio shield (<https://www.pjrc.com/store/teensy3_audio.html>) that is capable of stereo output, as demonstrated previously (Solari, Sviatkó, Laszlovsky, Hegedüs, & Hangya, 2018).

To attach the prop shield to the Teensy 3.2, we used 14x1 double insulator pins, and then fed the output to a speaker, as demonstrated in Figure 2B. The prop shield can power speakers with resistances up to 8 ohms (https://www.pjrc.com/store/prop\_shield.html). We also directed digital outputs from the Teensy to activate a light concomitant with the sound, and a puff as an aversive stimulus following each sound/light combination. Meanwhile, digital pulses were programmed to occur during every frame, which could be used to trigger image captures, for example.

In this case, we utilized “elapsedMicros” in order to reliably time all of the experimental events. “elapsedMicros” objects serve as time incrementers, that increment time at the microsecond time scale beginning every time that its value is set to zero. Every 50 ms, this codecalled a main function that updated the status of the digital pins associated with the “puff” and the light, and updated the amplitude of the 9500 Hz sine wave (amplitudes were set to 0.05 during audio stimulus time periods, and 0 elsewhere). Also, at the termination of a trial, this function incremented the trial number. Finally, a 1 ms digital pulse was delivered via another pin to instantiate a theoretical camera trigger. The speaker, camera, puff, and light source can be attached to the microcontroller using simple coaxial cables with SMA connectors, as shown in Figure 1A. The same programming environment (PlatformIO on top of Atom) was utilized, and functions such as “fastPinMode” and “fastDigitalWrite” were utilized to decrease latency.

In order to begin experiments with the Teensy, we wrote in MATLAB a simple graphical user interface that can be used on a desktop or laptop. With this, a user enters the length of the each trial and the number of trials desired. The PC or laptop sends this information over a USB connection to the Teensy, which in turn reports information about the experiment, in particular the frames during which the tone is on, the puff is on, or the light is on, and the experimental and trial times (in milliseconds) at the beginning of each IntervalTimer function call.

In our proof-of-concept experiment (Figure 3), the puff, tone, and camera trigger pins were all attached to and were recorded by the same external device (TDT RZ5D) at 3051.76 Hz for the puff and camera trigger pins, and 24414.0625 Hz for the tone pin. The tone pin was measured directly (not through the amplifier). We performed a mock-recording consisting of 50 trials of 20 seconds length each, where sound and light output pins were programmed to turned on 11.1 seconds into each trial for 700 ms, and the pin used to generate the aversive puff stimulus was turned at 12.05 seconds into each trial for 100 ms.

In order to measure latency (Figure 4Bi and iii), we acquired the timing of the camera digital pulse, according to the TDT system, that corresponds to the exact frame during which the audio signal was turned on. We then acquired the timing of either the puff pin onset or the timing of the onset of the audio signal. In order to measure the onset of the audio signal, we took the raw recording and high-pass filtered the signal using a 6th-order Butterworth filter, a bandpass frequency of 1000 Hz, and a “zero-phase digital filter” (MATLAB command “filtfilt”). Then, we took the absolute value of the Hilbert transform of the filtered signal to acquire an amplitude envelope. After finding the amplitude envelope, we found those values that exceeded a value of 0.005. The first time point that the amplitude crossed this threshold was considered the tone onset, and the next time point that dropped below this threshold was considered the tone termination.

*Statistics*

Linear models were constructed using the “fitlm” function in MATLAB 2017b. Theoretical timings, to which measured timings were compared, were each taken to be timings beginning at 0 seconds in equal increments of 50 milliseconds for both experiments.

**Results/Discussion**

Microcontrollers such as Arduino UNOs, with their user-friendly interface and low cost, have gained popularity in neuroscience research (D'Ausilio, 2012; Chen & Li, 2017; Micallef, Takahashi, Larkum, & Palmer, 2017). However, the Arduino UNO is somewhat limited in that it does not have true analog output. Further, while the Arduino UNO has several useful timing libraries, it lacks the IntervalTimer function, which in particular is optimal for precise control of experiments and precise acquisition of experimental data. This function takes as input a single main function and the time, in microseconds, desired between calls to this function. The Teensy 3.2 (<https://www.pjrc.com/store/teensy32.html>) is a newly developed microcontroller that not only has analog output and a comprehensive Audio library, but also has the capability to use the IntervalTimer function. Therefore, to maximize the flexibility of experimental design and maintain high accuracy, we utilized the Teensy 3.2 instead of the Arduino UNO.

*Motion tracking using ADNS-9800 sensors*

To demonstrate the flexibility of this device for both experimental control and data acquisition in conjunction with frame-wise image triggering, we constructed two separate and commonly utilized experimental setups both built upon a Teensy 3.2. In the first (Figure 1Ai and 1Aii), we constructed a device that monitors and records motor data from a three-dimensional treadmill at a fixed interval and delivers highly regular, brief digital pulses to, for example, an external device such as a scientific CMOS camera. As shown in Table 1, the cost of specialty components for this experimental design is quite low, totaling less than $80. Other commonly used components such as wire, solder, and tools are common in most labs , but are widely available. In addition to a Teensy, to detect motion, this system utilizes two ADNS-9800 laser motion sensor boards, which are also very affordable components (as shown in Table 1) and will be explained in depth below.

To measure motion, we constructed a “three-dimensional treadmill” setup, initially proposed by Dombeck, Khabbaz, Collman, Adelman, & Tank (2007) and utilized elsewhere (Aranov & Tank, 2014). In this setting, the mouse is fitted with a head plate and imaging window, and is suspended atop a Styrofoam ball that is supported by compressed air (Figure 1Aii). This type of imaging offers easily correctable in-plane jitter, as well as a setting in which mouse must apply similar forces to begin or to terminate a motor sequence as it would in a freely-moving setting (Dombeck, Khabbaz, Collman, Adelman, & Tank, 2007). Generally, two LED motion sensors are fit at the equator of the Styrofoam ball at an angle of 90 degrees, which provides the experimenter with linear movement in the X-Y plane, as well as rotational information. Such designs can obtain motor information from readings from the LED sensors via LabView (Aranov & Tank, 2014; Dombeck, Khabbaz, Collman, Adelman, & Tank, 2007) which, though a comprehensive and intuitive piece of software, is expensive.

We reconstructed this three-dimensional treadmill design utilizing a Teensy. Using a microcontroller allowed us to interface directly with ADNS-9800 sensor boards. As previously mentioned, these sensor boards are inexpensive and the sensors themselves are an improvement in many aspects over the sensors present in standard computer mice which were used in the previous studies (Aranov & Tank, 2014; Dombeck, Khabbaz, Collman, Adelman, & Tank, 2007). For example, they are highly sensitive and have high maximum sampling rates, with a maximum read rate of 12000 frames per second (thus accommodating the temporal requirements of faster imaging environments), and maximum resolution of 8200 counts per inch (<https://datasheet.octopart.com/ADNS-9800-Avago-datasheet-10666463.pdf>). Further, accumulated displacements can be stored in the sensors between readings, because ADNS-9800 sensors store motion data in 16 bits instead of the more standard 8 bits.

In order to use these motion-sensors, we utilized a class-based ADNS-9800 library. This library and these sensors highlight the benefits of working with low-cost and open source software: because of the ubiquity of Arduino microcontrollers, a large hobbyist community has developed and contributed software and devices that have the potential to be useful to the research community and accelerate the development of novel experimental design. For example, the low-cost ADNS-9800 sensor boards are fashioned by a very small company started by a hobbyist (https://www.tindie.com/stores/jkicklighter/). Useful add-ons and libraries are commonplace, and both contribute to the flexibility of these widely available microcontrollers.

With these sensors, we read displacements and converted them directly to micrometer displacements using the internal calibration of the sensors. Because of the simplicity of the ADNS-9800 library and example experimental design setup built alongside, building a usable design is straightforward. Proper wiring is also simple and is demonstrated in Figure 2B. The connections demonstrated using dotted lines can be replaced with jumper wires or sturdier, longer lasting wire. No knowledge of electrical circuits is necessary; this does not require any external capacitors or resistors, for example.

This system offers a simple and inexpensive method of tracking mouse movement with high fidelity and temporal accuracy while maintaining alignment with imaging data. As can be seen in Figure 3A, the velocity that we calculate falls into the range of previously reported mouse velocity with similar setups (see, for example, (Dombeck, Khabbaz, Collman, Adelman, & Tank, 2007)), and we are capable of seeing large variation in the mouse’s motor output. Further, the displacement measurements are recorded with high regularity. In Figure 3B, we also see that digital pulses administered at 50 ms increments closely track the theoretical times, biased in slope by an exceedingly small amount (approximately 28.9 microseconds per sample).

To verify that this bias in slope was not due to the frequency of the IntervalTimer, we repeated recordings that were 5 minutes long each, each using the same script except with a 500 microsecond delay between the beginning of the digital pulse and end of the digital pulse. These all had very similar biases, at 28.3 microseconds per second for the 20 Hz recording, and 28.4 microseconds per second for the 50 Hz and 100 Hz recordings, respectively. A much similar bias in timing was previously reported previously using an Arduino UNO: with repeated sampling of single 900 ms long TTL pulses with 100 ms inter-pulse intervals, the average length of time between sequential pulses was 1000.6 milliseconds (D'Ausilio, 2012). Though the code utilized by that experiment differs from ours, it does illustrate the precision and low bias of the Teensy combined with the IntervalTimer function. In addition, it underscores the utility of the Teensy for continual frame-capture triggering instead of aligning a camera only to the beginning of a trial or experiment, particularly over the course of a longer recording session (Micallef, Takahashi, Larkum, & Palmer, 2017).

*Trace conditioning*

In the second experiment (Figure 1B and 2A), we constructed a device capable of running a simple trace conditioning experiment, where an experimenter trains a mouse to associate a predictive toneand/or light to a subsequent puff of air as an unconditioned aversive stimulus.Our trace conditioning experimental design mimics a setup previously reported by our lab (Mohammed, et al., 2016). Here, we set up the Teensy to perform such an experiment, and recorded from the relevant pins. In addition to the Teensy 3.2, we needed only 2 additional specialty components in addition to a speaker, as shown in Table 2: a plug and play hardware amplifier (prop shield) to amplify the analog output from the Teensy 3.2, which can then drive speakers of both 4 and 8 ohms, and three sets of 14x1 double insulated pins for connecting the Teensy to the prop shield. In total, this setup costs approximately $40, excluding general equipment.

Imaging can be performed simultaneously by turning on and off a given pin during each frame. In this mimic experiment, we recorded the timings of each of these triggers and compared them to the theoretical timings with samples spaced at exactly 50ms apart, as shown in Figure 4A. Like the motion experimental design, the measured timings were very similar to the theoretical timings, biased by approximately 30 microseconds per sample. We looked at light onset timing, light length, interstimulus length, and puff length in Figure 4B as well. All were very consistent over the 50 trials, with standard deviations well under 1 milliseconds, showing that even with including a continuous audio output, it doesn’t alter the accuracy or increase the bias of experimentation with a Teensy.

**Conclusion**

We introduce two inexpensive and highly accurate experimental paradigms both constructed around a Teensy 3.2 microcontroller. In the first, we utilize highly accurate ADNS-9800 gaming sensors, for which exists a user-friendly library. The Teensy is capable of utilizing these sensors and reporting movement measurements while sending aligned, temporally regular and precise digital pulses out of another digital pin. This is particularly useful in an imaging paradigm, where one would set a camera to external capture mode and send output associated with movement simultaneously paired with accurate camera trigger. This experiment also highlights the benefits of these inexpensive microcontrollers: with such a large user-base and an intuitive programming language, many novel software libraries are available for Arduino that wouldn’t be otherwise. The Teensy utilizes the same programming environment, thus benefitting from the ubiquitous use of Arduinos while adding several features.

We also demonstrate a setup built to implement a trace conditioning paradigm. In addition to requiring accurate alignment of imaging with behavior, operant conditioning paradigms need reliable stimulus timing. In this setting, repetition of stimulus and response must occur in a highly regular temporal fashion in order for a mouse to learn and in order for the neuronal response to be consistent. This illustrates the ability of the Teensy to orchestrate different classes of output—analog and digital, both long and short pulses—simultaneously and with high temporal accuracy while simultaneously sending out regular digital pulses to control an image capturing device. It also highlights the ability of this device to simultaneously produce an analog output, in particular to generate a sound, while performing other actions. As previously stated, a major advantage of the Teensy 3.2 over other microcontrollers such as the Arduino is the fact that it can output a true analog signal, whereas the Arduino UNO, for example, is capable only of outputting pulse-width modulated signals. This opens a venue for many experimental additions, particularly the addition of sound, without the need of extra devices such as resistors and capacitors to create an analog-like signal. Rather, the Teensy 3.2 simply needs to be soldered on to a paired hardware module (prop shield), and less in-depth knowledge about electronic circuits is necessary. In addition, it has a built-in “Audio” library that simplifies sound synthesis, reading, and mixing, all at 44.1 kHz which is stereo quality.

An important discovery during development ofthis system is that realization of a slight linear drift of the Teensy processing clock. This drift is linear in nature , which makes it simple to calibrate out if actual (sub-µs precision to real world timing is essential). Further, it actually underscores the the necessity of a central controller for precise acquisition and total experimental control. Synchronizing different devices only by a single pulse at the start of an experiment can lead to problems when trying to acquire motor output or deliver some experimental stimulus and examine cellular behavior with high temporal accuracy. Initiating experimental events from a high-level source, such as directly from a PC, can introduce timing jitter due to the multitude of tasks that a PC must attend to at any given point in time. For example, a recent calcium imaging study in the striatum finds additional neurological structure related to motor activity on very short timescales, suggesting that poor timing resolution could potentially lead to incorrect inferences (Markowitz, et al., 2018).

Further, with concomitant imaging, one must also align tasks to imaging data after the fact, or face substantial variability in frame spacing. As explained previously (D'Ausilio, 2012; Solari, Sviatkó, Laszlovsky, Hegedüs, & Hangya, 2018), using a microcontroller such as an Arduino or Teensy 3.2 circumvents the issue of imprecise timing of behavioral events. We note that in addition, synchronizing camera triggers with experimental events circumvents the need of post-hoc image alignment.

In conclusion, Arduino UNO and the Teensy 3.2 both potentially fulfill these requirements, though an additional timing function, the “IntervalTimer”, makes the Teensy 3.2 better suited for the particular task of delivering the equally spaced, regular digital pulses needed for triggering image capture. Finallythe precision and utility of the Teensy microcontroller, in conjunction with an available Audio library and the IntervalTimer function, make this a user-friendly, easily adaptable, accurate, and precise tool for different experimental designs in neuroscience in general, and particularly for imaging studies.

**Figures**

**Figure 1.** Diagrams of the two experimental device setups, a floating, 3D treadmill with two sensors for recording motor output (A) and a tone/light and puff classical conditioning setup. **A** This experimental design consists of a Teensy 3.2 connected to two ADNS-9800 sensors and a CMOS camera, via serial-peripheral interfaces and a coaxial cable via SMA connectors, respectively. Every 50 milliseconds, a digital pulse triggers the CMOS camera to capture an image while simultaneously acquiring motor data from both ADNS sensors and sending them via a USB to a PC. The PC initiates each experiment by sending serial data consisting of the length of the experiment and imaging frequency to the Teensy. **B** This experimental design constitutes a classic classical-conditioning paradigm. The user specifies via MATLAB or via a different interface the length and number of experimental trials. This information is sent via a USB to the Teensy 3.2, which initiates the experiment. In each trial, the Teensy initiates a 9500 Hz tone at 44.1 kHz while turning on a light. These stimuli are followed by an air puff, also delivered via the Teensy. In order to generate a sound loud enough for the speaker, the Teensy is soldered to a prop-shield, which contains an amplifier. The Teensy 3.2 sends time stamps, trial, and stimulus information via the USB back to the PC.

**Figure 2. A.** A schematic demonstrating the wiring connections between a Teensy 3.2, prop shield, and an external speaker. Dotted lines indicate solid connections. All connections between the Teensy 3.2 and prop shield were made using 14x1 double insulated pins, and the output to the speaker from the prop shield was made using regular wire and a coaxial cable. Some extraneous and unused pins on the Teensy and the prop shield were not included in this diagram. **B.** A schematic demonstrating the wiring of Teensy to two ADNS-9800 sensors via serial peripheral interface connections (SPIs). Solid dots at intersections between lines indicate connections. Some unused pins on the Teensy 3.2 were not included in this schematic.

**Figure 3.** **A.** Part of a sample 10 minute recording session during which a head-fixed animal was allowed to run on the three-dimensional treadmill. Shown in **Figure 1A**. The mouse’s average speed was 7.1 + 6.9 cm/s, with a maximum velocity of 47.0 cm/s, within ranges reported elsewhere. **B.** Times of digital pulses sent by the Teensy 3.2 as measured internally by the Teensy, vs times of the digital pulses as measured by an external device. Green indicates linear model, and in black are experimental data, down-sampled by a factor of 200. The linear model estimates a slope of 1.000028927 + .000000005 (t(11997)= 2.0381e+08, p < 0.001, R2=1; intercept = 0.000107 + 0.000002, t(11997) = 63.243, p < 0.001), indicating an excellent fit and very nearly a 1:1 correspondence of time stamps.

**Figure 4.** **A.** Timing of the digital pulses as measured by the Teensy 3.2 in the tone/light-puff setup versus timing as measured by an external device. These measurements have a correspondence near 1:1 (R2=1, slope: 1.0000336 + 4e-10, t(14998)=2.35e+09, p<0.001). **B.** Timing measured by the teensy for (i) and by the TDT system for (ii-iv) over the course of fifty trials; (i) shows the latency between the theoretical onset of the tone and the measured timing of the tone as measured by the TDT device (mean=7.6 + 0.9 ms, range=2.9 ms); (ii) shows the consistency of the length of tone intervals across all trials (mean=700 + 1 ms, range=2.9 ms); (iii) shows the consistency of the latency of the tone stimulus interval, as measured by the TDT sytem (mean= -0.004 + 0.012 ms, range=0.04 ms); (iv) shows the consistency of the length of the puff across all trials (mean = 100.03+0.02 ms). (all + std).

**Tables**

Table 1. Specialty components necessary to build a motor output system

|  |  |  |  |
| --- | --- | --- | --- |
| **Part name** | **Website** | **Part number** | **Cost per unit** |
| Teensy 3.2 | https://www.pjrc.com/store/teensy32.html | TEENSY32 | $19.80 |
| ADNS-9800 sensors | https://www.tindie.com/products/jkicklighter/adns-9800-laser-motion-sensor/ | None | $27.50 |

Table 2. Specialty components necessary to build a tone/light-puff system.

|  |  |  |  |
| --- | --- | --- | --- |
| **Part name** | **Website** | **Part number** | **Cost per unit** |
| Teensy 3.2 | https://www.pjrc.com/store/teensy32.html | TEENSY32 | $19.80 |
| 14x1 Double insulator pins | https://www.pjrc.com/store/header\_14x1\_d.html | HEADER\_14x1\_D | $0.85 |
| Prop shield | https://www.pjrc.com/store/prop\_shield.html | PROP\_SHIELD | $19.50 |

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