**Abstract**

Background: Systems neuroscience experiments often require the integration of precisely timed data acquisition and behavioral monitoring. While specialized commercial systems have been designed to meet various needs of data acquisition and device control, they often fail to offer flexibility to interface with new instruments and variable behavioral experimental designs.

New method: We developed a Teensy 3.2 microcontroller-based interface that offers high-speed, precisely timed behavioral data acquisition and digital and analog outputs for controlling sCMOS cameras and other devices.

Results: We demonstrate the flexibility and the temporal precision of the Teensy interface in two experimental settings. We first used the Teensy interface for reliable recordings of an animal’s directional movement on a spherical treadmill, while delivering repeated digital pulses that can be used to control image acquisition from a sCMOS camera. In another example, we used the Teensy interface to control temporally precise delivery of an auditory stimulus and a gentle eye puff in a trace conditioning eye blink behavioral paradigm, while delivering repeated digital pulses to initiate camera image acquisition.

Comparison with existing methods: This interface allows high-speed and temporally precise digital data acquisition and device control during diverse behavioral experiments.

Conclusion: The Teensy interface, consisting of a Teensy 3.2 and custom software functions, provides a temporally precise, low-cost, and flexible platform to integrate sCMOS camera control into behavioral experiments.

**Keywords:** *Teensy, Arduino, microcontroller, sCMOS camera, open-source, spherical treadmill, ADNS-9800 gaming sensor*

**1. Introduction**

Recent advances in sCMOS camera technology and genetically encoded neural activity indicators enable large scale fluorescence imaging of thousands of individual cells’ activity during behavior (Mohammed et al., 2016; Nguyen et al., 2016). One key technical aspect of neural network analysis during behavior is temporal precision, where neural activities need to be precisely aligned with behavioral features. However, it has been difficult to easily integrate sCMOS cameras, deployed in large scale calcium imaging studies, with devices needed to monitor and control behavioral experiments.

Traditional Analog/Digital interfaces are often operated by programs, such as MATLAB, that offer a wide range of applications. However, using high-level programming in MATLAB or other PC-based programs can lead to undesired temporal delays, as the PC operating system needs to balance the demands of many system operations at once. Further, lower-level implementations of sCMOS camera control, for example using optimized MATLAB programming or a graphical program such as LabVIEW, have steep learning curves. Thus, a method of sCMOS camera control that involves high precision and easy programmability is highly desirable, particularly for broad audiences. This includes those whose primary focus is biology or those who are not proficient programmers.

Over the last decade, microcontrollers traditionally marketed to hobbyists have gained popularity across a variety of scientific fields (D'Ausilio, 2012; Sanders and Kepecs, 2014; Husain et al., 2016; Chen and Li, 2017). For example, Arduino microcontrollers have recently been integrated into two-photon imaging experiments (Wilms and Hausser, 2015; Takahashi et al., 2016; Micallef et al., 2017). Microcontrollers are small, low-cost, and capable of delivering digital outputs with microsecond time precision. Arduino, which utilizes user-friendly, open-source software functions, was the first major microcontroller to gain substantial popularity. Recently, Teensy 3.2 microcontrollers were developed, which have all the key features of the current version of the standard Arduino microcontroller (Arduino Uno Rev3), as well as the additional feature of delivering analog output. Teensy microcontrollers utilize the same open-source Arduino software environment, and remain easy to program (D'Ausilio, 2012). Because of the simplicity of microcontrollers and their temporal precisions, microcontrollers represent an attractive solution to precisely record data and monitor experimental progress.

Here, we demonstrate and characterize a flexible Teensy 3.2-based interface for temporally precise data acquisition and delivery of analog and digital signals, during a voluntary movement tracking experiment and trace conditioning eye blink learning experiment. The Teensy interface can deliver digital pulses with microsecond precision to initiate individual image frame capture using the camera’s external trigger settings at a desired frequency, while simultaneously collecting animal behavioral data. We also demonstrate the ability of the Teensy interface to generate analog sound waveforms to drive a speaker for a trace conditioning eye blink experiment. Together, these results demonstrate that the Teensy interface, consisting of a Teensy microcontroller and a set of custom software functions, offers a flexible, accurate, and user-friendly environment for imaging experiments during behavior.

**2. Methods**

The Boston University Institutional Animal Care and Use Committee approved all animal procedures and the methods followed the approved guidelines. Two 8-12 week old female C57BL/6 mice were used in this study (Taconic; Hudson, NY). These mice were first injected with AAV9-Syn-GCaMP6f.WPRE.SV40 virus, which was acquired from the University of Pennsylvania Vector Core (titer ~6e12 GC/ml). This virus was injected stereotaxically, with 250 nL of virus into the CA1 region (AP: –2 mm, ML: 1.4 mm, DV: –1.6 mm) at 40 µl/min. To accomplish this, we used a 10 nL syringe (World Precision Instruments, Sarasota, FL) and a 33 gauge needle (NF33BL; World Precision Instruments, Sarasota, FL). The injection rate was commanded by a microsyringe pump (UltraMicroPump3–4; World Precision Instruments, Sarasota, FL). After the mice recovered, they were fitted with a stainless steel cannula (OD: 0.317 in., ID: 0.236 in., height 2 mm) along with an attached coverslip (size 0; OD: 3 mm), attached via UV-curable adhesive (Norland Products). This constituted the imaging window. Cortical tissue was aspirated and the window was placed on top of CA1. An aluminum head-plate was then affixed to the skull. Mice were trained on an eye-blink task in an identical fashion to Mohammed et al. (2016), including using the same MATLAB code in conjunction with a National Instruments Data Acquisition board (USB 6259, National Instruments, Austin, TX).

2.1 *Construction of Teensy 3.2 boards*

The two experimental hardware designs are shown in Figure 1. The specialty components required to build these designs are shown in Tables 1 and 2. In both experiments, a Teensy 3.2 (PJRC.COM, LLC, part #: TEENSY32) (Figure 1A), or a Teensy 3.2 soldered to a prop shield (PJRC.COM, part #: PROP\_SHIELD) (Figure 1B), is mounted on top of a standard printed circuit board (PCB) (for example: Digi-Key, part #: V2010-ND) via standard female headers (such as SparkFun Electronics, PRT-00115). Female headers were then soldered to the PCB for stability. Output from the Teensy was directed from pins on the female headers to standard SMA connectors (such as: Digi-Key, part # CON-SMA-EDGE-S-ND) via 22 gauge wires (for example: Digi-Key, part #1528-1743-ND). Coaxial cables were then attached to the SMA connectors on the PCB to connect the Teensy to external devices. The Teensy was connected to a computer via a standard USB-microUSB cable (for example: Digi-Key, part # AE11229-ND). To easily upload code to the Teensy, we used PlatformIO (<https://platformio.org/>), an add-on to the widely-used Atom text editor (<https://atom.io/>), instead of the default Arduino programming environment. Code for each of the two experimental settings were uploaded separately to the Teensy prior to each experiment. To turn digital pins on and off, and also to change their modes to either “input” or “output”, we used a slightly modified version of the DigitalIO library provided by PlatformIO (version 1.0.0; currently maintained at: <https://github.com/greiman/DigitalIO>). To easily set experiment-specific parameters for the Teensy, such as sampling frequencies, trial numbers and trial length, and the length of an experiment, we developed simple MATLAB graphical user interfaces, one for each experiment. Thus, a user plugs the Teensy in via a USB cable to the computer, waits for it to initialize (i.e. run a “setup” function), and then can begin a recording session by pressing “Start” in the user interface. Unless otherwise specified, the Teensy was attached to a sCMOS camera, though not turned on or capturing images.

2.2 *Motion tracking experiment*

In this experiment, we performed motion tracking using two ADNS-9800 gaming sensors (Tindie, part: “[NS-9800 Laser Motion Sensor](https://www.tindie.com/products/jkicklighter/adns-9800-laser-motion-sensor/)”, see Table 1), while delivering digital pulses that can be used to trigger a sCMOS camera for image capture every 50 ms. The overall design of this experiment is shown in Figure 1A. A mouse was positioned on top of a buoyant Styrofoam ball floated by house air as described previously (Dombeck et al., 2007). Two ADNS-9800 gaming sensors were positioned at the equator of the Styrofoam ball, at an angle of approximately 75 degrees from one another. ADNS-9800 sensor boards are low cost, and can measure up to 8200 counts per inch, allowing for sensitive measurement of mouse movement relative to other tracking devices. For example, standard computer mice, such as the Logitech M100 (Logitech, PN: 910-001601), measure up to 1000 counts per inch, making the ADNS-9800 sensor, at its highest setting, over 8 times more precise. For the counts per inch setting of the sensor in our experiments, we used a value of 3400 counts per inch.

The total distance travelled by the mouse at any 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). We also acquired readings in the “x” direction from both sensors, which can be used to calculate rotation. Velocity was computed as the distance divided by the time between two adjacent readings, as measured by the Teensy. These two sensors were connected to the Teensy via simple serial peripheral interface (SPI) connections with insulated 22 gauge wires as shown in Figure 2A.

In order to control the beginning of each imaging session, we developed a unique GUI in MATLAB. This GUI allows a user to specify a filename, the length of each trial, and the sampling rate. The user plugs the Teensy 3.2 into the computer via a USB, and waits a moment for the device to initialize. Then, the user can press “Start” on the GUI in order to start the experiment. In addition, we developed an alternative GUI and accompanying Teensy code that includes a “Stop” button, which allows a user to stop and restart an experiment from the GUI. Both MATLAB GUIs store in the respective PC the Teensy-reported time stamp of each frame, the Teensy-reported duration of each frame, and the displacement in the X- and Y- directions for both sensors for each time frame.

To control experimental timing, we utilized the “IntervalTimer” function, unique to the standard Teensy library, which can repeatedly call a function at specified intervals. We set the “IntervalTimer” to be 50,000 microseconds (50 ms) or 20 Hz in our experiment. Using IntervalTimer, we repeatedly called a function that sent the accumulated displacement of the motion sensor readings to the attached PC. We acquired the x and y displacement readings from each sensor with freely available functions on GitHub (<https://github.com/markbucklin/NavigationSensor>), which read accumulated displacement from the “motion burst” register of each sensor. After reading the motion sensor, a digital “on” pulse that lasted for 1 ms was sent out of a digital pin designed to initiate an image frame capture from a sCMOS camera. To characterize the temporal precision of different digital pulses generated by the custom script using the “IntervalTimer” function, we recorded the digital outputs with a commercial system (Tucker Davis Technologies RZ5D (TDT RZ5D)) at 3051.76 Hz. One of the mice was run for 10 minutes on this treadmill, though no imaging data was collected.

2.3 *Trace conditioning eye blink experiment*

In this experiment, a Teensy was programmed to deliver outputs capable of eliciting a sound and initiating an eye puff, while delivering digital pulses that can be used to trigger a sCMOS camera for image capture every 50 ms. The overall design of this experiment is shown in Figure 1B. To deliver an audible sound through the Teensy, we used a Teensy prop shield module (PJRC.COM, LLC., part #: PROP\_SHIELD) to amplify analog output (shown in Figure 2B as pin A14). This add-on component can drive speakers with resistances up to 8 ohms. The prop shield was soldered to the bottom of the Teensy with 14x1 double insulator pins (PJRC.COM, LLC., part #: HEADER\_14x1\_D), and the output was connected to a speaker, as shown in Figure 2B. The Teensy was then mounted onto the female headers separated by the prop shield, as shown in Figure 1B. The camera and air valve for the eye puff were attached to the microcontroller through coaxial cables (Figures 1B and 2B), and the speaker was connected with 22 gauge wire to the prop shield.

We used the Teensy Audio library function “AudioSynthWaveformSine” to generate tones. This function continuously outputs a sine wave with a sampling rate of 44.1 kHz from the analog pin. We first initialized the tone, in this case a 9500 Hz sine wave, at the beginning of each experiment, but set the amplitude to “0”, so that the tone was off. At the desired time, we switched the amplitude to 0.05 (out of a maximum of 1) to generate an audible tone. The value of 0.05 generated a tone of approximately 75 dB with our amplifier and speaker settings.

We used the “elapsedMicros” function to control the timing of the experiment. elapsedMicros offers precise timing like “IntervalTimer”, and additionally allows for simultaneous use of the Audio library. This experiment is trial-based, and each trial consisted of an 11.1 second long baseline period, a 700ms long tone, a 250ms long delay period, a 100ms long puff period, and a 7.85 second long post-puff period. Using an “elapsedMicros” timer, we repeatedly called a function that updated the status of each digital and analog output every 50 ms based on the trial structure of the task, and then turned on the digital output directed to the sCMOS camera for 1ms every 50ms. These features were all hardcoded into the Teensy code.

In order to control the beginning of each imaging session, we developed a GUI in MATLAB similar to that used for the motion tracking experiment. This GUI allows a user to specify a filename, the length of each trial, and the total number of trials in the session, and saves the Teensy-reported trial times, experimental times, and status of all relevant pins. The specific characteristics of the tone and puff were hard-coded into the Teensy script. The user plugs the Teensy 3.2 into the computer via a USB, and then waits a moment for the device to initialize. Then, the user can press “Start” on the GUI in order to start the experiment. As with the motion tracking experiment, we developed a secondary GUI and accompanying Teensy code that includes a “Stop” button as well, which allows a user to stop and begin a new experiment from the GUI. MATLAB stores in the attached PC the Teensy-reported time stamp of each frame relative to the session and relative to the beginning of the trial, the trial number, and indicator variables (1s or 0s) which correspond to whether or not the sound or puff is on.

To characterize the temporal precision of different digital pulses generated by the custom scripts, we recorded the digital outputs with a commercial system (Tucker Davis Technologies RZ5D (TDT RZ5D)) at 3051.76 Hz, and the analog sine wave output at 24414.0625 Hz without additional amplification. To determine the onset of the analog audio signal, the unamplified analog output from the Teensy was first high-pass filtered at 1 kHz using a 6th-order, zero-phase Butterworth digital filter (MATLAB command “filtfilt”). We then estimated the instantaneous amplitude of the 9500 Hz sine wave at each time point using the Hilbert transform of the filtered signal. The first time point where the amplitude rose above 0.005 was considered the onset of the analog signal, and the subsequent time point where it dropped below 0.005 was considered the offset. To compare the onset of the analog signal to the timing of digital pulses, we utilized the continuous voltage output from the digital pin for consistency. To acquire the digital pulse onset from the continuous signal, we thresholded this continuous voltage output at a value of 1 V, and took the first time point where the continuous voltage exceeded 1 V to be the digital pulse onset.

2.3.2 *Imaging*

Imaging was accomplished using the same custom microscope used by Mohammad et al. (Mohammed et al., 2016). To demonstrate that digital pulses delivered by the Teensy to the sCMOS camera were capable of eliciting frame capture, we recorded a video session of the hippocampus of a mouse during this trace conditioning eye-blink experiment. This particular recording session consisted of 40 trials, each lasting 20 seconds. Videos were stored as TIFF files, each of a duration of 2047 frames and an approximate size of 4 GB.

We next processed these videos using a standard processing pipeline, modified very slightly from one implemented previously (Mohammed et al., 2016). Briefly, videos were first filtered using a homomorphic filter, which functions as a spatial high-pass filter, and then motion corrected using a cross-correlation method. Finally, the videos were background-subtracted as follows. First, a minimum projection of the first video in each video sequence was obtained and smoothed. This smoothed image was then subtracted from each video recorded in this session and a constant was added in order to prevent pixel values from reaching values below zero. Next, a projection of the range of every pixel in the minimum-subtracted first video was obtained. This projection was smoothed, and pixels in the smoothed projection that had values less than the mean smoothed range across all pixels in the first video were identified. The values of these pixels were spatially averaged for each frame in the recording session, and the averaged value for each frame was subtracted from all of the raw pixel values in the respective frame to acquire a background-subtracted frame. The median of the averaged background pixel values across all frames in the first video was obtained, and this constant value was added to every frame in every video to prevent values from reaching below 0 before converting the values to uint16.

Regions of interest, which are presumptive neurons, were identified using the max-minus-mean projection from the first 3 processed videos using a previously established method, ACSAT (Shen et al., 2018). Traces were acquired by averaging together each pixel within each region of interest, and ∆F/F values were computed for each trace by subtracting the trace fluorescence mean and then dividing by the trace fluorescence mean.

2.3.3 *Trace conditioning eye blink experiment with two tones*

We next designed an expanded user interface and accompanying Teensy code for use with the experimental hardware design in Figures 1B and 2B in order to perform the same trace conditioning experiment with 2 tones. The user interface allows the user to specify the length of each trial, the number of trials, and the timings of two tones as well as the timing of a gentle puff following the second tone. The user can further specify the amplitudes of each of the two tones, their frequencies and their durations, and the duration of the gentle puff.

In our example recording, we utilized a 2000 Hz sound followed by an 8000 Hz sound, and recorded both using the aforementioned TDT system. We specified amplitudes of 0.05 and 0.2 (out of a maximum of 1), respectively. To identify the beginnings and ends of the tones, we used 6th-order bandpass Butterworth filters, using lower and upper frequency cutoffs of 1000 and 3000 for the 2000 Hz tone and 7000 and 9000 for the 8000 Hz tone. We identified onsets as the first time point where the amplitude of the Hilbert analytic signal of these filtered signals exceeded 0.025 for the lower amplitude 2000 Hz signal and 0.05 for the higher amplitude 8000 Hz signal. The signal corresponding to the “puff” output was not recorded or monitored for this demonstration.

2.4 *Statistics*

Statistics were performed in MATLAB. Linear models were constructed using the “fitlm” function in MATLAB 2017b. Root mean squared error was computed by taking the square root of the mean of the squared residuals from a linear model.

2.5 Code availability

All code is located at GitHub (<https://github.com/mfromano/micro-control>), which will be made public upon publication.

**3. Results**

Microcontrollers such as Arduino microcontrollers have gained popularity in neuroscience research, and provide a user-friendly interface, open-source software environment, low cost, and in particular, a high flexibility for integration with different devices (D'Ausilio, 2012; Chen and Li, 2017; Micallef et al., 2017). Recently, the Teensy 3.2 has been developed, which has an analog output, a major improvement over the popular Arduino Uno. Teensy devices also have a comprehensive Audio library, as well as the “IntervalTimer” and the “elapsedMicros” functions capable of generating precisely timed events repeatedly. Here, we present a Teensy-based interface to integrate frame-by-frame image capture with behavioral experimental control and data acquisition.

3.1 *Motion tracking experiment*

In this experiment (Figure 3A), we recorded a mouse running on a spherical treadmill for 10 minutes. Motion data was acquired at 20 Hz concomitantly with digital outputs that can be used to trigger individual image frame capture from a sCMOS camera.

To measure locomotion from awake head fixed mice, we used the Teensy interface to record from two ADNS-9800 motion sensors (Figures 1A and 2A). We calculated the velocity of the mouse, which averaged 2.16 + 4.46 cm/s over the 10 minute period (mean + std, n=12000 time points/10 minutes) with a maximum velocity of 35.9 cm/s, in general agreement with velocities reported for head-fixed mice running on a spherical treadmill (Dombeck et al., 2007).

To characterize the temporal precision of the Teensy interface, we measured the timing of the Teensy digital output, and compared it to the theoretical 20 Hz signal using a linear model. We found that digital outputs have a near-perfect linear relationship with the theoretical signal (Figure 3B). However, we noted a 28.9 µs per second positive drift, resulting in an actual frequency of 19.999 Hz instead of 20.000 Hz, which is shown in detail in Figure 3C. To further examine whether this small timing drift depends upon the frequency of data acquisition or the timing of the digital outputs, we performed 5 minute long recording sessions without a live mouse at 20, 50, and 100 Hz, and a 0.5ms long digital pulse designed to trigger image capture from the camera. We found that the actual frequencies were 19.999, 49.999, and 99.997 Hz, respectively. These all correspond to an approximately 30 µs delay per second, suggesting that the timing drift is independent of the data acquisition rate and may reflect the processor timing of the Teensy microcontroller. However, because motion sensor data are monitored with respect to the Teensy’s timing, the animal’s locomotion data readings remain precisely aligned to the time when image frame capture occurs.

Having assessed the timing of the digital output, we next quantified its temporal variation. We calculated the root mean squared error (RMSE) of the difference between the recorded timing of each digital pulse and the times predicted from the linear model. The RMSE was 42.7 µs, computed manually. Together, these results demonstrate that the Teensy interface timed by the “IntervalTimer” function can be used to generate digital pulses for precise image frame capture during behavioral experiments, while maintaining alignment of imaging data with behavioral parameters.

3.2 *Trace conditioning eye blink behavioral experiment*

In a second experiment, we reconfigured the Teensy interface for a trace conditioning eye blink learning experiment (Figure 1B and 2B), where a mouse can be trained to associate a conditioned stimulus (700ms long tone) with a subsequent unconditioned stimulus (a 100ms long gentle eye puff), separated by a brief memory trace time window (250ms). This experiment consisted of 50 trials, each lasting 20 seconds. We first characterize the temporal precision of the Teensy interface in a manner similar to that described in the motion tracking experiment. We recorded the timings of the digital pulses generated to trigger each image frame capture (Figure 4A), and detected a 33.4 microsecond delay per second. Thus, in this experiment, the Teensy interface has an actual frequency of 19.999 Hz instead of 20.000 Hz, identical to that observed in the motion tracking experiment. This delay is demonstrated in Figure 4C. The RMSE of the Teensy interface is 13.3 µs computed manually, also similar to that observed in the motion tracking experiment.

We then characterized the precision of multiple digital outputs by calculating the time difference between the digital pulses generated to drive eye puff versus the sCMOS camera (Figure 4Biii). We found that there was nearly no temporal difference between the onset of these two digital outputs (-0.004 + 0.012 ms,mean + std, n=50 digital pulses). Similarly, the duration of the puff digital pulse was within 0.03 ms of the commanded duration of 100ms (Figure 4Biv) (100.03+0.02 ms (mean + std, n=50 digital pulses).

We next characterized the temporal precision of the analog output generated by the Teensy in the context of a trace conditioning experiment with a single tone. We measured the analog output of the Teensy with the commercial TDT RZ5D recording device sampled at 24414.0625 Hz. Since analog outputs were generated together with the onset of the digital outputs designed to trigger camera image frame capture, we calculated the time difference between the onset of the analog output and the onset of the digital pulse (Figure 4Bi, for details see Methods). We found that the analog output lagged the digital output by 7.6 + 0.9 milliseconds (mean + std, n=50 pulses, Figure 4Bi). This delay is comparable to that reported using a different configuration of the Teensy to play a sound (Solari et al., 2018). The duration of the tone remained equal to 700 + 1 ms, (mean +/- std, n=50 digital/analog pulses Figure 4Bii), equivalent to the commanded duration of 700ms. Together, these results demonstrate that the Teensy interface, timed by the “elapsedMicros” function, is capable of generating digital and analog output with microsecond temporal precision.

To further examine whether this delay was related to our implementation of the Audio library or from writing to the analog pin itself, we directly generated an analog pulse without the Audio library from the analog pin using the Arduino command “analogWrite(A14, 4050)”. “A14” corresponds to the analog pin, and 4050 is a relative voltage level large enough to be recorded as a pulse by the TDT RZ5D system. We initiated 50 trials consisting of 50 millisecond long pulses through a digital pin and through the analog pin. Pulses to these two pins were programmed to occur near-simultaneously. We found that the analog output lagged the digital output by 0.8 + 5.8 µs (mean + std, n=50 trials), suggesting that writing to the analog pin cannot account for the auditory signal delay generated through the Audio library. Thus the delay is due to the specific implementation of the audio library, and future changes to the Audio library could improve the temporal precision.

Finally, we conducted a single-tone, trace conditioning eye-blink experiment calcium fluorescence while imaging from the hippocampus of a mouse. We detected ROIs (putative neurons), and a zoomed-in max-minus-mean projection map demonstrating an overlay of the neurons identified is shown in Figure 5A. For each neuron, we then computed the average fluorescence centered around each tone onset, shown in Figure 5B. These neurons were sorted by their mean fluorescence during the inter-stimulus interval. Clearly, we can see that certain neurons are highly responsive to this tone. A slight movement artifact coinciding with the onset of the puff also demonstrates that the alignment of the video recording with the tone puff is precise. Thus, we were able to recapitulate data previously acquired using a National Instruments Data Acquisition (NI DAQ) board (USB 6259) by Mohammad et al. (2016).

3.3 *Trace conditioning eye blink behavioral experiment with two tones*

We next tested the experimental paradigm in which we sequentially generated two tones of different amplitudes and frequencies over the course of 10 trials. Their overall time course is shown in Figure 6A, and the amplitude envelopes of two example low and high pitched tones are shown in Figure 6B, with examples of the actual bandpassed signals shown in Figures 6C and 6D. The latency of these two tones was similar to that of the single-tone experiment and did not differ from one another, with the low-frequency sound having a latency of 7.5 + 0.8 ms (+/- std) and the high-frequency sound having a latency of 7.1 + 0.9 ms (Wilcoxon rank-sum test, p=0.385, ranksum = 93). This demonstrates the flexibility of a single Teensy interface to command experiments with multiple audio stimuli.

**4. Conclusion and Discussion**

In both the motion tracking and single-tone trace conditioning tone-puff experiments, the Teensy interface generated precisely timed digital pulses that can be used to control individual frame capture from a sCMOS camera at 20Hz. We detected a small drift of approximately 30 µs per second, suggesting an actual frequency of 19.999 Hz instead of the commanded 20Hz. This small 0.003% drift of the Teensy processing clock is linear, and can thus be calibrated if desired. This finding underscores the importance of having a highly precise central timer in each experiment.

Synchronizing different devices such as sCMOS cameras only at the start of an experiment can lead to undesired temporal drifts, particularly in long experiments. Using a central timer instead of parallel, asynchronous controllers, however, is currently challenging. While MATLAB programs can be used to control experimental timing when integrating a sCMOS camera into a behavioral paradigm, they may introduce timing problems due to the concurrent demands of many PC system operations. For example, MATLAB offers a Data Acquisition Toolbox. Learning how to optimize experiments using this toolbox is challenging, and poor implementations that utilize high-level timers (e.g. the “tic”/“toc” functions), while easy to program, can result in timing jitter. Such timing jitter may have a significant impact depending on the study, especially when millisecond time scale resolution is desired in systems neuroscience experiments. This is one of the central challenges with incorporating sCMOS cameras into experimental design.

Using a lower-level interface such as LabVIEW or using advanced MATLAB programming to control timing potentially obviate timing jitter, but both have steep learning curves, are proprietary, and have high costs. In contrast, with the Arduino programming environment it is simple to program basic experiments and because it is open source, there are many libraries on sites such as GitHub already available for adoption and use in experiments. Teensy boards are also inexpensive, and accompanying software and programming environments are free to download. Further, the GUIs that we have designed allow users of diverse scientific backgrounds to design a basic tone-puff experiment or motion control experiment without having to implement any Arduino programming at all.

Temporal accuracy is often important for animal behavioral training. For example, a precisely timed conditioned stimulus (tone) and unconditioned stimulus (puff) are important for animals to build association in trace conditioning eye blink experiments. We demonstrate that the Teensy interface can accurately generate multiple digital pulses to drive different devices, including the tone, the puff and the sCMOS camera. Additionally, we demonstrate that the Teensy interface precisely delivered longer duration digital and analog pulses, such as the tone that lasted for 700ms in the single tone trace conditioning eye blink experiment. These results demonstrate that the Teensy interface is a viable, inexpensive alternative that is also able to simultaneously capture imaging data using our simple software functions.

A major advantage of the Teensy over Arduino Uno microcontrollers is its ability to generate a true, 12 bit analog signal. While Arduino Uno microcontrollers can generate an analog-like signal via pulse-width modulation, this output is a square wave. We used the Teensy interface to deliver an auditory stimulus through the built-in Audio library, and our analog output showed a 7.6 ms delay during delivery of the single tone. This small delay is due in large part to the implementation of the Audio library. It is possible that other ways of utilizing the analog output would allow the generation of more temporally precise audio signals. However, altering the amplitude of a single sine wave via the Audio library is easy to implement, utilizing only a few lines of code within a single script.

4.1 *Conclusion*

We demonstrate a Teensy 3.2 interface capable of integrating a sCMOS camera into two behavioral experimental settings. In one setting, the Teensy interface simultaneously generates digital pulses that can be directed for individual frame capture from a sCMOS camera, while simultaneously tracking an animal’s locomotion using recently developed high precision ADNS-9800 gaming sensors. The easy integration of the sCMOS camera and the ADNS-9800 sensors illustrates the flexibility of the Teensy interface in designing experiments that require novel instrumentation. In the second experimental setting, we demonstrate that the Teensy interface, in conjunction with a prop shield, is capable of generating both analog and digital outputs with precise timing during an eye blink trace conditioning experiment. We characterized two timer functions, “IntervalTimer” and “elapsedMicros”, both of which offered equivalent microsecond temporal precision, and “elapsedMicros” additionally allows access to the Audio library. Thus the Teensy interface, a Teensy 3.2 and custom software functions, provides a user-friendly, easily adaptable, and temporally precise platform for integrating sCMOS cameras into behavioral experimental designs. This Teensy interface can be immediately adopted for the motion tracking and the trace conditioning eye blink behavioral experiments demonstrated here, or can be customized for other types of behavioral experiments where sCMOS camera-based imaging is desired.

**5. Figures**

**Figure 1.** Diagrams of the two experimental device arrangements using a Teensy interface. **A** Motion tracking experiment design. This design consists of a Teensy 3.2 connected to two ADNS-9800 sensors via serial-peripheral interfaces, and a sCMOS camera through a coaxial cable. Every 50 milliseconds, a digital pulse was sent to initiate an image frame capture from a sCMOS camera. Simultaneously, the Teensy interface acquired motion data from both ADNS sensors and sent them to a PC via a USB. **B** Trace conditioning eye blink experiment design. This design consists of consists of a Teensy 3.2 connected to a speaker through a prop-shield that contains an amplifier. Every 50 milliseconds, a digital pulse was sent to initiate an image frame capture from a sCMOS camera. Simultaneously, the Teensy interface generated digital pulses to generate air puff and updated the status of the analog output to generate audio signals, and sent the timing of these signals to a PC via a USB.

**Figure 2.** Electrical wiring schematics for the motion tracking experiment and the trace conditioning eye blink experiment **A.** The schematic of the wiring of a Teensy 3.2 to two ADNS-9800 sensors via serial peripheral interface connections (SPIs). Solid dots at intersections between dotted lines indicate electrical connections. Unused pins on the Teensy were not included in this schematic. The Teensy’s ground pin was connected to both AGround and DGround pins (analog and digital ground) on both ADNS-9800 sensors. The D11 pin (D = digital) was connected to both MOSI (“Master-Out, Slave-In”) pins, the D12 pin was connected to both MISO pins (“Master-In, Slave-Out”), the D13 pin was connected to both SCK pins (SPI Clock), and the 3.3V pin was connected to both Vin (voltage in) pins on the ADNS-9800 sensors. Finally, pins D20 and D21 were connected individually to each SS pin (Slave Select) on the ADNS-9800 sensors. The DAC pin (digital to analog converter or the analog output pin) is also shown. **B** The schematic of the wiring of a Teensy 3.2, a prop shield, and an external speaker. Dotted lines indicate connections. Connections between the Teensy and prop shield were made using 14x1 double insulated pins according to the manufacturer’s instruction (https://www.pjrc.com/store/prop\_shield.html), and the prop shield audio output was connected to the speaker using 22 gauge wire. We highlight that that the Teensy DAC pin is connected to the “Audio In” pin on the prop shield, both of which are labeled. Additional pins utilized by the prop shield for amplification were also labeled.

**Figure 3.** Temporal precision of the digital outputs in the motion tracking experiment. **A** Example recording of a head-fixed mouse running on the spherical treadmill. **B** Timing of digital pulses generated by the Teensy interface vs theoretical times of the digital pulses at exactly 20 Hz. Red indicates linear model fit of experimental data, and black are experimental data downsampled by a factor of 200 for visualization. The linear model estimates a slope of 1.000028937 + 0.000000002 (t(11998)= 4.9e+08, p < 0.001, R2=1; intercept = 0.0007593 + 0.0000007, t(11998) = 1.1e+03, p < 0.001). **C** (i)The best fit line from (B) shown in red, plotted against a theoretical line measured at exactly 20 Hz with no temporal drift (blue). (ii) and (iii) are zoomed in windows demonstrating the close fit at the beginning of the session and the gradual drift that becomes very evident toward the end of the session.

**Figure 4.** Temporal precision of the digital and analog outputs in the trace conditioning eye blink experiment. **A** Timing of the digital pulses generated by the Teensy interface vs theoretical times of the digital pulses at exactly 20 Hz. Linear model fit for experimental data is shown in red, and in black are experimental data down-sampled by a factor of 200 for visualization. (R2=1, slope: 1.0000334 + 0 (to machine precision), t(19998)=infinite, p<0.001). **B.** Timing of the analog output directed to the prop shield to generate an amplified auditory stimulus (i-ii) and the digital output directed to device to generate eye puff (iii-iv), both measured over 50 trials. (i) the difference between the onset of the analog output and the onset of the corresponding camera-directed digital pulse (mean=7.6 + 0.9 ms, range=2.9 ms); (ii) the duration of the auditory stimulus across all trials (mean=700 + 1 ms, range=2.9 ms, n=50 trials); (iii) the difference between the puff digital pulse and the camera-directed digital pulse, (mean= -0.004 + 0.012 ms, range=0.04 ms); (iv) the duration of the puff digital pulse (100.03+0.02 ms, mean + std, n=50 trials). **C.**(i)The best fit line from (B) shown in red, plotted against a theoretical line at exactly 20 Hz with no temporal drift (blue). (ii) and (iii) are zoomed in windows demonstrating the close fit at the beginning of the session and the gradual drift that becomes very evident toward the end of the session.

**Figure 5.** A demonstration of a hippocampal recording using an sCMOS camera during the trace conditioning eye blink experiment. **A.** An overlay of the identified ROIs plotted on top of a max-minus-mean image over the course of the first 3 videos in a recording session. **B.** The mean, trial-averaged response for each of the identified ROIs, centered at tone onset. The three black lines indicate tone start, tone termination, and puff start, from left to right, respectively. ROIs are sorted by their mean, trial-averaged fluorescence between the end of the tone and start of the puff.

**Figure 6.** Demonstration of the audio signal from the tw- tone trace conditioning eye blink experiment. **A.** An example, zoomed-out recording of two tones, 2000 Hz (lower amplitude) and 8000 Hz (higher amplitude), plotted over a series of 10 trials each 20 seconds in length. **B.** Amplitudes of both the 2000 Hz and 8000 Hz signals over a sample time course. **C.** Example recorded waveform of a 2000 Hz signal over the course of 0.0169 seconds. **D.** Example recorded waveform of an 8000 Hz signal over the same amount of time.

**6. 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/ | ADNS-9800 Laser Motion Sensor | $27.50 |

Table 2. Specialty components necessary to build a tone-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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M.F.R. performed data analysis. M.F.R. and H.J.G. conducted the motion tracking experiment. M.F.R. conducted the trace conditioning eye blink experiment. M.F.R., M.B., and D.R.M. wrote the software. M.F.R., M.B., D.R.M., and R.K. contributed to the Teensy interface conceptualization. M.F.R., H.J.G., and X.H. wrote the manuscript. X.H. supervised the study. The authors would also like to acknowledge Thomas Romano for helpful conversations, and users “Theremingenieur” and “PaulStoffregen” from the PJRC forums (<https://forum.pjrc.com/>) for responding to questions relating to the trace eye blink conditioning experiment.

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