**Title: A Teensy microcontroller-based interface for optical imaging camera control during behavioral experiments**

**Running title: Teensy interface for optical imaging**

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**Highlights**

* **Utilize a Teensy 3.2-based interface to deliver precisely timed digital pulses to initiate frame capture using a sCMOS camera**
* **Demonstrate temporally precise behavioral data acquisition using Teensy 3.2 interface combined with concurrent sCMOS camera control**
* **Demonstrate experimental control of combined analog (sound waveforms) and digital pulses delivered simultaneously with camera-control**

**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 highlight 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 puff in a trace conditioning behavioral paradigm, while delivering repeated digital pulses to initiate camera image acquisition.

Comparison with existing methods: This Teensy interface allows high-speed and temporally precise imaging analysis during diverse behavioral experiments.

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

**Keywords:** *Teensy, Arduino, microcontroller, sCMOS camera, open-source, spherical treadmill*

**1. Introduction**

Recent advances in sCMOS camera technology and genetically encoded calcium sensors enable large scale fluorescence imaging of thousands of individual cells’ activity during behavior (Barbera, et al. 2016, Klaus, et al. 2017, Markowitz, et al. 2018, Mohammed, et al. 2016). One key technical aspect of neural network analysis during behavior is the temporal precision, where neural activities need to be precisely aligned with behavioral features (Solari, et al. 2018). 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 which often work independently. Traditional Analog/Digital interfaces are often operated by programs, such as MATLAB, that offer a wide range of applications. However, using 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.

Over the last decade, microcontrollers marketed to hobbyists have gained popularity across a variety of scientific fields (Chen and Li 2017, D'Ausilio 2012, Husain, Hadad and Zainal Alam 2016, Sanders and Kepecs 2014). Microcontrollers are small, low-cost, and capable of delivering digital outputs with microsecond time precision. The 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 standard Arduino Uno microcontroller (the current version of which is the Rev3 (Arduino, Arduino Uno Rev3)), as well as the additional feature of delivering analog output. These microcontrollers utilize the same open-source Arduino software environment, which is easy to program (D'Ausilio 2012). For example, Arduino devices or microcontrollers have recently been integrated into two-photon imaging experiments for image acquisition control (Micallef, et al. 2017, Takahashi, et al. 2016, Wilms and Häusser 2015). One way to perform precisely timed acquisition of each image frame is to trigger the acquisition of each frame independently, while simultaneously acquiring behavioral data. To do this, one can use the external trigger setting of the camera as demonstrated previously (Micallef, et al. 2017). Because of the simplicity of microcontrollers and their temporal precisions, microcontrollers represent an attractive solution to precisely record digital 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 a trace conditioning learning experiment. The Teensy interface can deliver digital pulses with microsecond precision to initiate individual image frame capture 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 an auditory 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**

2.1 *Construction of Teensy boards*

The two experimental 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 printed circuit board 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 the DigitalIO library (<https://github.com/greiman/DigitalIO>), which decreases the amount of time spent performing each of these actions. To easily set experiment specific parameters for the Teensy, such as the sampling frequency, the trial number and trial length, and the length of an experiment, we developed a simple MATLAB graphical user interface.

2.2 *Motion tracking experiment*

In this experiment, we performed motion tracking using two ADNS-9800 gaming sensors (<https://www.tindie.com/products/jkicklighter/adns-9800-laser-motion-sensor/>, 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 sphere, at an angle of approximately 75 degrees from one another. For the counts per inch setting of the sensor, which determines the sensitivity of the sensors to external movement, we used a value of 3400 counts per inch. Thus, 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 subsequent readings, as measured by the Teensy. These two sensors were connected to a Teensy via simple serial peripheral interface (SPI) connections with insulated 22 gauge wires as shown in Figure 2A.

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 interval between calls to this function to 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 a 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.

2.3 *Trace eye blink conditioning 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 a 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 (Figure 1B), 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 initially 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 to generate analog output. 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 “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 turned on the digital output directed to the sCMOS camera for 1ms every 50ms.

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. For digital outputs, the TDT system automatically identified digital pulse onsets. To determine the onset of the 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. When comparing the timing of the analog output to the timing of digital pulses, we utilized the continuous voltage output from the digital channel for consistency. To acquire digital pulse onset times, 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.

2.4 *Statistics*

Statistics were performed in MATLAB. Linear models were constructed using the “fitlm” function in MATLAB 2017b.

**3. Results**

Microcontrollers such as Arduinos have gained popularity in neuroscience research due to their user-friendly interface, open-source software environment, and their flexibility of device integration (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 function, which is 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 example experiment (Figure 3A), we recorded a mouse running on the 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).

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, the LED of a standard computer mouse, the Logitech M100 (Logitech, PN: 910-001601), measures up to 1000 counts per inch, making the ADNS-9800 sensor over 8 times more precise at its highest setting. For these experiments we affixed ADNS-9800 sensors were to the housing of a Styrofoam ball spherical treadmill and wired them to the Teensy as demonstrated in Figure 2A.

We calculated the velocity of the mouse, which averaged 2.16 + 4.46 cm/s over the 10 minute period (mean + std) 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, Howe and Dombeck 2016)).

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. 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 internal frequency of the Teensy microcontroller. However, because behavior is monitored with respect to the Teensy’s timing, behavior remains temporally precise relative to the frame triggers.

Having assessed the timing of digital outputs, we next wanted to assess its variation in timing across different output samples. We calculated the root mean squared error (RMSE) of the difference between the recorded timing of each digital output sample and the predicted times from the linear model. This roughly corresponds to the standard deviation of the errors of the model fit. The root mean squared error is 38.3 microseconds, indicating that the digital output has microsecond-level precision. Together, these results demonstrate that the Teensy interface using 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 eye blink conditioning behavioral experiment*

In a second experiment, we reconfigured the Teensy interface for a trace conditioning learning experiment (Figure 1B and 2B). This experiment consisted of 50 trials, each lasting 20 seconds. Through these trials, 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).

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 found 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. The RMSE of the Teensy interface is 13.3 us, 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 devices for the eye puff and the sCMOS camera (Figure 4Bii). We found that there was nearly no temporal difference between the onset of these two digital outputs (-0.004 + 0.012ms (mean + std, n=50 digital pulses)). Similarly, the duration of the puff digital pulse was 100.03+0.02 ms (mean + std, n=50 digital pulses), within 0.03ms of the commanded duration of 100ms.

We next characterized the temporal precision of the analog output generated by the Teensy. We measured the analog output waveforms 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 used 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 camera digital pulse by 7.6 + 0.9 milliseconds (mean +/- std, n=50 digital/analog pulses, Figure 4Bi). This delay is could be related to the manner in which we generated the tone—by altering the amplitude of an ongoing sine wave—which we utilized for its simplicity. 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.

**4. Conclusion and Discussion**

In both 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 necessity of having a highly precise central timer in each experiment. Synchronizing different devices at the start of an experiment can lead to different degrees of temporal drifts, particularly in long experiments, and while MATLAB or other PC-based programs can be programmed to control experimental timing, they may introduce timing jitter due to the demands of many PC system operations. Such timing jitter can have a significant impact depending on the study, especially neuronal processing, which is often at the time scale of milliseconds.

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

A major advantage of the Teensy over many other microcontrollers is its ability to generate a true, 12 bit analog signal. While Arduino UNO microcontrollers can generate an analog-like sinusoids via pulse-width modulation, this output has the shape of a square wave. We used the Teensy interface to deliver an auditory stimulus through the built-in Audio library, and our analog output showed just a 7.6ms delay. This small delay could be due in part to the way in which we generated a tone utilizing the Audio library. It is also possible there are other ways of utilizing the analog output that could offer even more precision. However, altering the amplitude of a single sine wave with this library simultaneously with outputting a digital pulse for image capture is easy to implement, utilizing only a few lines of code within a single script. Also, our delay is comparable to sound onset delays reported using a different configuration of the Teensy to play a sound (Solari, et al. 2018). Thus, this Teensy interface allows easy implementation for diverse experimental designs, including the ones needing analog outputs.

4.1 *Conclusion*

We demonstrate a Teensy 3.2 interface 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 experiment, 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 a 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 to generate analog output. Thus the Teensy interface, a Teensy 3.2 and custom functions, provides a user-friendly, easily adaptable, and temporally precise tool for integrating sCMOS cameras into behavioral experimental designs. This Teensy interface can be immediately adopted for the motion tracking and trace conditioning behavioral experiments demonstrated here, or customized for other types of behavioral experiments, where sCMOS camera-based imaging is desired.

**5. Figures**

**Figure 1.** Diagrams of the two complete experimental device arrangements for using a Teensy interface. **A** A floating, spherical treadmill setup for a motion tracking experiment. This experimental 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 eye blink conditioning design. In order to generate a sound through the speaker, the Teensy is soldered to a prop-shield, which contains an amplifier. The Teensy 3.2 sends self-monitored time stamps, trial, and stimulus information (audio, and puff pin states) via the USB back to the PC. In each trial, the Teensy generates a 9500 Hz tone at a sampling rate of 44.1 kHz. The tone stimulus is followed by a gentle air puff 100 ms in duration.

**Figure 2.** Electrical wiring schematics for both the motion tracking and trace eye blink conditioning experiments **A.** A 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 lines indicate connections. Some unused pins on the Teensy 3.2 were not included in this schematic. **B** A schematic of the electrical connections between a Teensy 3.2, a prop shield, and an external speaker. Dotted lines indicate 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 constructed using 22 gauge wire. Some extraneous and unused pins on the Teensy and the prop shield were not included in this diagram.

**Figure 3.** Experimental design and movement data from animal tracking experiment. **A** Example motion recording of a head-fixed mouse running on the spherical treadmill shown in Figure 1A. **B** Times of digital pulses generated by the Teensy vs theoretical times of the digital pulses at exactly 20 Hz. Red indicates linear model prediction, and black are experimental data, down-sampled by a factor of 200. The linear model estimates a slope of 1.000028937 + 0.000000002 (t(11998)= 4.95e+08, p < 0.001, R2=1; intercept = 0.000759 + 0.000007, t(11998) = 1084.7, p < 0.001).

**Figure 4.** Temporal precision of the digital outputs in the trace conditioning tone-puff setup. **A** Times of digital pulses generated by the Teensy vs theoretical times of the digital pulses at exactly 20 Hz. Linear model fit 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 digital output directed to device to generate eye puff (iii-iv), both measured over fifty 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 tone 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).

**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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