Automated Analysis of Freezing Behavior

Freezing was quantified using video-based recognition software. The software was written in Borland Delphi Personal 7 (Borland Software Corporation, Austin, TX) using the digital video capturing component (DSPack version 2.3.4). All behavioral sessions were video-recorded. Live images from an analog infra-red sensitive CCD camera (Circuit Specialists, Mesa, AZ) were digitized and encoded into MPEG-2 format (720 x 480 pixels, 2.5 MBits/s) by a hardware encoder (WinTV-PVR-USB2, Hauppauge Computer Works, Inc., Hauppauge, NY). The video files were stored on a PC computer. Files were trimmed using the native MPEG editor VideoReDo (DRD Systems, Inc. Mendham, NJ) and stored on DVD data discs for future analysis.

Detection and Measurement of Movement. Video images were captured at 5 Hz. Movement was measured by calculating the number of pixels that changed between consecutive video images (see Fig. 1B and C). Pixels were analyzed further when the absolute change in the pixel grayscale luminosity between successive images was above a user-defined difference threshold. In the present study, the difference threshold was always set to 20 steps, which covered 7–8% of the full 256 grayscale-luminosity range. The two-dimensional (2D) map of differences was written to a black and white bitmap (Fig. 1D).

The 2D bitmap was further processed with a spatial filter to eliminate "salt-and-pepper" noise. Each pixel at the center 3 x 3 matrix was classified as indicating movement only when a user-defined number of the neighboring pixels was also above the difference threshold. In the present study, each pixel was evaluated based on all 8 immediately-adjacent pixels. Next, pixels on the differential bitmap were colored red or yellow depending, respectively, on whether they did or did not satisfy the criteria for movement. This pseudo-colored map was then superimposed on the live video image.

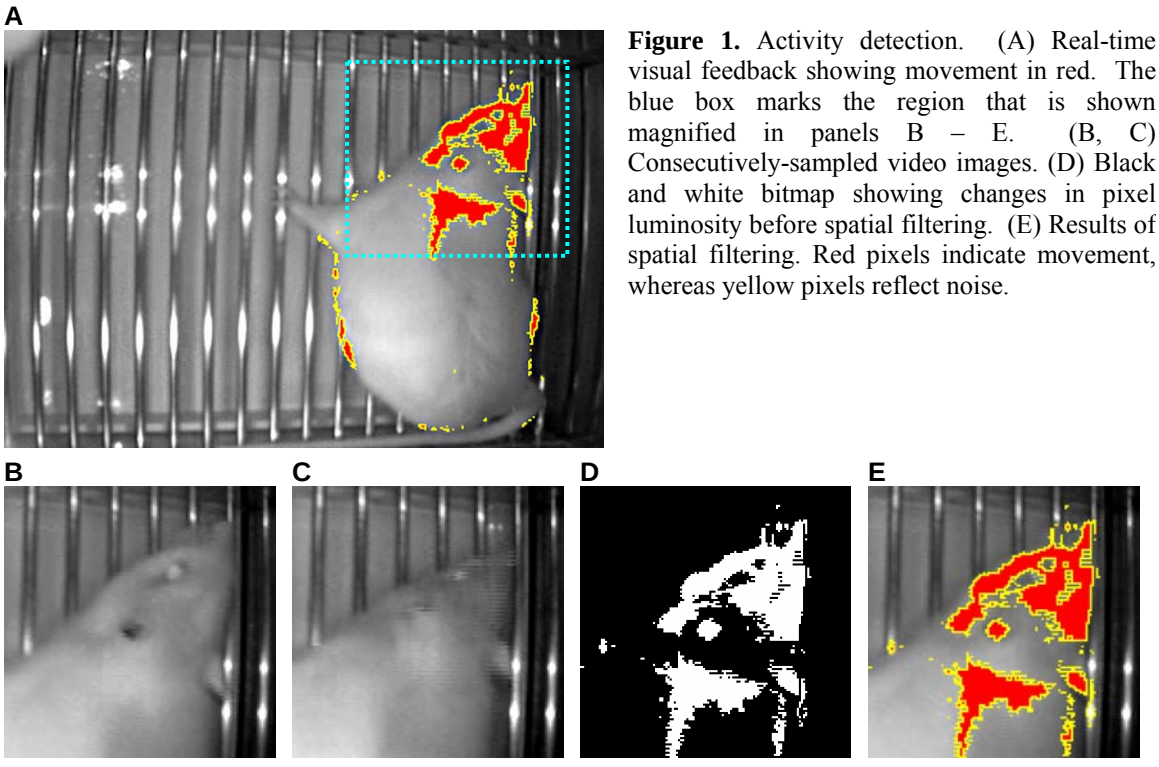

Figure 1. Activity detection. (A) Real-time visual feedback showing movement in red. The blue box marks the region that is shown magnified in panels B – E. (B, C) Consecutively-sampled video images. (D) Black and white bitmap showing changes in pixel luminosity before spatial filtering. (E) Results of spatial filtering. Red pixels indicate movement, whereas yellow pixels reflect noise.

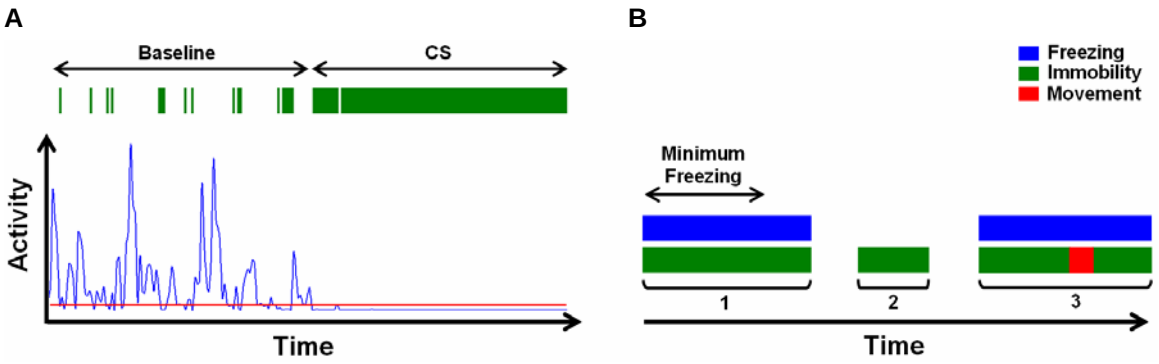

Figure 2. Converting immobility to freezing. (A) The amount activity (the number of pixel changes) is plotted as a function of time. The red horizontal line represents the immobility threshold. During the baseline period (before the CS presentation) there were occasional instances of immobility that were too brief to be counted as episodes of freezing (see panel B). By contrast, immobility greatly increased during the CS presentation. (B) Instances of immobility were converted to measurements of freezing. The first immobility period (labeled 1) was longer than the user-defined minimum and was therefore classified as freezing. The second immobility period (labeled 2) was shorter than the minimum duration and was therefore not classified as freezing. The third period (labeled 3) includes two episodes of immobility, separated by a brief movement. When such movements were briefer than a user-defined value, they were reclassified as instances of immobility.

The red pixels in Figure 1 show head and body movements (Figs. 1A and D). These composite images provided real-time visual feedback that was used for three purposes. First, they revealed the effect of the number of pixels that were included in the spatial filter. Second, they disclosed the effect of variations in the selected value of the difference threshold. Third, these images showed the consequence of the definition of freezing, which is explained in the next section. Once the parameters and definitions were set (for a given chamber and lighting conditions), the analysis proceeded automatically for any number of recorded sessions.

Operational Definition of Freezing Behavior. Figure 2A shows a time series of the number of pixels that were determined to have changed. The operational definition of freezing entailed three considerations. First, freezing was defined by an upper limit on the amount of allowable movement (based on the number of changed pixels). This threshold number of pixel changes was set to exclude small movements associated with breathing or the emission of ultrasonic vocalizations. In the present study, this number was always set to 20 pixels. This movement threshold is depicted by the red line in Figure 2A. The green bars in Figure 2A mark epochs that were classified as instances of immobility. As indicated, the rat was quite active before but not after the CS onset.

Second, freezing was defined to include a minimum duration of immobility (always 3 s in the present experiment). Part 1 of Figure 2B shows an episode of immobility that lasted longer than the minimum. The entire duration was therefore classified as an instance of freezing, which is denoted by the blue bar. Part 2 of Figure 2B depicts an episode of immobility that did not reach the minimum duration and was therefore not classified as freezing, noted by the absence of a blue bar.

Third, the definition of freezing ignored movements whose duration did not exceed a user-defined maximum. Part 3 of Figure 2B shows two immobility periods (green bars) that were interrupted by activity that was too brief to be taken into account using manual scoring. When such movements were briefer than a user-defined maximum ($\leq$ 0.6 s in the present experiment), they were reclassified as periods of immobility. The red bar in part 3 of Figure 2B shows a brief

movement that was reclassified as a period of immobility. After this reclassification, the total duration of immobility satisfied the 3-s rule for freezing, as depicted by the blue bar. A separate experiment showed that the machine analysis closely approximated manual scoring (see Materials and Methods).