Converting Qualitative Observations of Tardigrade Freezing Survival into Quantitative Metrics via Image Processing

Introduction to Image Processing and Analysis (71254)

Date: 09/02/2025

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Introduction:

Tardigrades are a group of microscopic animals that have captured the curiosity of scientists, both for their abilities to survive environmental extremes and for their high potential to be developed as a model system for a wide range of biological questions[1] and translational applications[2]. Under certain environmental stresses, tardigrades evade harm by entering various forms of protective dormancy known as cryptobiosis or encystment, as seen in desiccation, osmotic stress, or radiation[3]. As one especially striking example of survival, researchers found that a species of Antarctic tardigrades Acutuncus antarcticus could be successfully revived from storage in the tun state (desiccated) at -30°C for over 30 years, with no addition of cryoprotectants or unique storage protocols. However, these forms of protective dormancy involving tun or cyst formation are not often seen in cases of cold-exposed tardigrades originating from less extreme habitats[4].

Instead, many species of cold-exposed tardigrades are believed to go into a physiological state known as cryobiosis, where active animals can survive low temperatures, and their overall morphology remains essentially unchanged compared to tun or cyst formation[5]. Supporting this claim, a recent ecological survey of microfauna in snow algae blooms in northern Japan indicated that physiologically active tardigrades were among the most abundant organisms, thus suggesting that tardigrades do not rely on deep dormancy in snowy environments. Likewise, a microbiome diversity survey conducted in British Columbia detected a high abundance of snow algae genetic material in tardigrade gut contents, indicating snow algae consumption by tardigrades during winter months[6]. Despite these observations indicating tardigrades' abilities to tolerate winter conditions, few studies have rigorously characterized the impact of cold on hydrated, active tardigrades, especially under ecologically relevant conditions where exposure time, temperature, and environmental ice-inoculators may vary. Methodological challenges in handling and scoring these unique microscopic animals—especially with the high accuracy and throughput needed to interrogate interactions between these crucial factors—have historically contributed to this gap in knowledge .

Low temperatures and freezing are undoubtedly significant selective pressures that tardigrades experience, given their global distribution in cold climates with seasonal or frequent subzero temperatures[7]. All tardigrade species require a film of liquid water in order to locomote, feed, and reproduce, and ice-formation threatens this requirement, presumably impacting essential elements of tardigrades' metabolic function, cellular integrity, and other temperature-dependent physiological processes[8]. As microscopic water-dwelling organisms, tardigrades are especially vulnerable to rapid shifts in ice formation. Yet, environmental factors impacting their survival in subzero temperatures and ice formation are poorly understood. To survive subzero temperatures while hydrated, tardigrades are believed to deploy one of two major cold tolerance strategies: freeze avoidance or freeze tolerance[5]. Organisms using freeze-avoidant strategies cannot survive internal ice formation and use colligative solutes such as glycerol or trehalose to suppress the freezing point of their body fluids or employ cryoprotective dehydration to avoid ice formation[9]. Freeze-tolerant organisms can survive extracellular or even intracellular ice formation and often do so by inoculating ice formation at relatively warm subzero temperatures using ice-nucleating proteins, resulting in ice formation

occurring more slowly[9]. It remains unknown if tardigrades produce their own antifreeze or ice-nucleating proteins or if they are influenced by the ice-nucleating proteins or agents from any neighboring microorganisms, such as bacteria or fungi that share their winter-time habitat.

Although several studies have attempted to describe the cold tolerance strategies of tardigrades using calorimetry, with the goal of detecting liquid-to-ice phase transitions in tardigrade samples, conclusions of the exact mode, plasticity, and robustness of cold tolerance remain ambiguous. Early calorimetric studies conducted on limno-terrestrial species Richtersius (Adorybiotus) coronifer and Amphibolus nebulosus detected relatively high ice-crystallization temperatures (-6 to -7°C) for tardigrades, suggesting freeze tolerance as the primary strategy to survive cold[10]. Likewise, these authors predicted that ice nucleators likely played an important role in modulating the higher-than-expected freezing temperatures for these tardigrade species. More recent studies on nine tardigrade species indicated that phase transitions occurred at much lower temperatures, with tardigrades experiencing supercooling points ranging from roughly -10°C to -24°C. However, most species survived ice formation well[11].

This study investigates the cold tolerance of the tardigrade species Hypsibius exemplaris, an emerging model organism in biological research. While previous research has focused on desiccation tolerance in this species, its cold tolerance strategies remain largely unexplored. This research addresses this gap by developing a novel, high-throughput method for assessing tardigrade mortality after cold exposure using the vital dye SYTOX Green, which stains only dead cells. This method offers improved accuracy and efficiency compared to traditional methods relying on visual assessment of locomotion, which can be confounded by dormancy or other non-lethal factors. The study examines the impact of various factors on cold tolerance, including temperature, duration of exposure, the presence of ice-nucleating bacteria, and thermal acclimation, aiming to elucidate the mechanisms underlying cold tolerance in tardigrades and establish H. exemplaris as a robust model for future comparative studies of stress tolerance across tardigrade species.

In addition to the established indirect assessment of cryo survival, a complementary, direct methodology was developed. This method employs a directional freezing apparatus to target and manipulate the freezing of individual tardigrades. Precise control over temperature decrement allows for observing internal ice formation within the tardigrade body fluids, a direct indicator of freezing. Post-thaw, complete recovery is determined by the resumption of pre-freezing activity levels. This direct visualization of internal ice nucleation and subsequent physiological recovery offers valuable insights into the cryotolerance mechanisms operating at the individual organismal level.

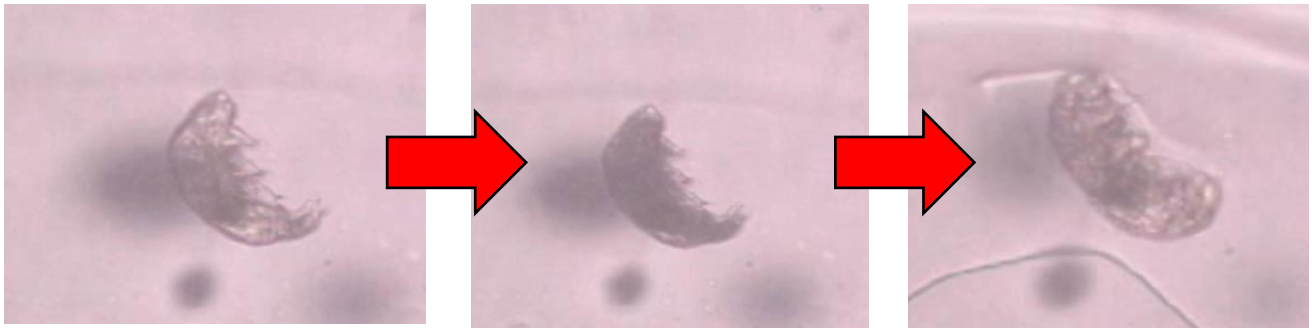
Although possessing notable advantages, this methodology yields qualitative rather than quantitative data. Consequently, developing an image-processing pipeline capable of converting these qualitative observations into quantifiable metrics is essential. Such a quantitative approach will permit inter-replicate comparisons and enable robust statistical analysis of the experimental outcomes.

An image-processing method was implemented to facilitate the quantification of qualitative observations. Deployed via a custom-built web interface to optimize computational resource

utilization, this method transforms microscopy images into graphical representations amenable to quantitative analysis and inter-experimental comparison. The underlying RGB-based image processing algorithm confers adaptability to diverse microscopy image formats, ensuring broad applicability across experimental paradigms.

Database:

The dataset was acquired in our lab during experimental manipulation of tardigrades, utilizing a microscope-mounted camera to capture 8-bit grayscale images at a frequency of 8 frames per second. Within these recordings, the freezing process is visually represented by a transient decrease in image intensity specifically within the region of the tardigrade, attributable to the attenuation of light transmission by the forming ice, which consequently reduces the photon flux reaching the camera sensor. This change in image intensity constitutes the focus of our quantitative analysis, the objective of which is to translate this visual observation into a quantifiable metric.



Methods:

A custom code was implemented to address this challenge and facilitate the accurate conversion of qualitative video observations into quantitative metrics. This code comprises the following procedural steps:

1. Receiving the video from the user
2. Video to Image:

   A Python script was developed to extract individual frames from a video file and store them as discrete image files within a designated directory. This script utilizes the os and moviepy libraries. The os library provides access to operating system functionalities, including directory creation and file system checks. The moviepy library enables the loading and processing of video files. The script defines a function, extract_frames, which accepts the video file path, output directory path, and frame extraction rate as arguments. This function creates the output directory if it does not already exist, loads the specified video file, extracts frames at the defined interval, saves the extracted frames as JPEG images within the designated output directory, and generates a success or error message upon completion of the process.
3. Segmentation of each image using K-means[12]:

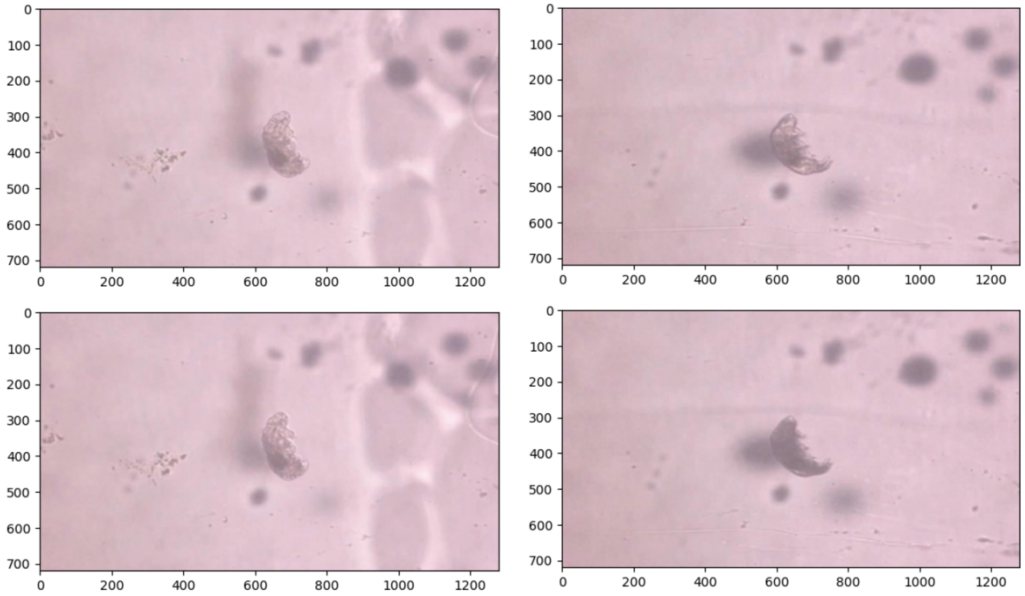
   K-means clustering, a common unsupervised machine learning technique in image processing, is employed here for image segmentation. This algorithm groups similar pixels based on features

such as color or intensity by iteratively assigning each pixel to the nearest of k randomly initialized cluster centers (centroids) and subsequently updating these centroids to reflect the average features of their assigned pixels. This iterative process continues until centroid stabilization, effectively partitioning the image into k segments. While k-means offers simplicity and efficiency, it necessitates a pre-defined number of clusters, exhibits sensitivity to initial centroid placement, and assumes approximately spherical cluster shapes, which can limit its effectiveness with complex morphologies or outlier data. In this application, the goal is to segment microscopy images into tardigrade and background regions. Given the approximately circular morphology of the tardigrade, k-means clustering is deemed a suitable and straightforward approach, and its characteristics are well-suited to the segmentation task.

4. Transforming Image Data into Graphical Representations:
   Image data were visualized graphically to represent the distribution and range of pixel values. This graphical representation facilitated the analysis and interpretation of image characteristics by providing a visual overview of all pixel intensities.

Results:

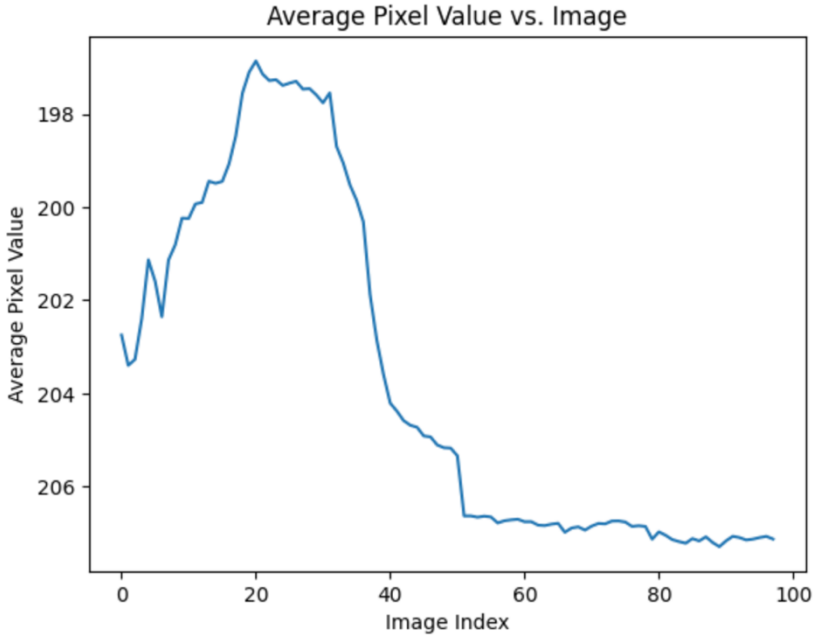
1. Video to Image:



2. The output of the values of each image after using the k-means algorithm:

```
[202.7465169986093, 203.39844906565972, 203.26943252368628,
202.40081705729168,
… ,
207.09593930844906, 207.0695334201389, 207.1281615306713]
```

3. Transforming Image Data into Graphical Representations:



Discussion and Conclusion:

This study provides compelling evidence of tardigrade cryotolerance, demonstrating their capacity to endure internal freezing, characterized by solidifying body fluids, and recover to full physiological function. While these initial findings offer significant qualitative insights into this phenomenon, the transition to quantitative data analysis became necessary to facilitate robust comparisons across experimental conditions and enable rigorous statistical evaluation. Such a quantitative approach is essential for deriving reliable conclusions and informing future research directions within this field.

A novel methodology was developed to translate qualitative observations from experimental video recordings into quantifiable metrics to address this need for quantitative data. This involved generating graphical representations of the freezing and thawing processes observed in the tardigrades. This process encompassed several key steps: (1) partitioning the video recordings into discrete image frames; (2) segmenting each frame to isolate the tardigrade and determine its gradient relative to the background; (3) recording the segmentation values for each frame; and (4) visualizing these values as a time-series graph. The duration of the post-thaw period required for the tardigrades to resume regular activity was then used as a quantitative measure of recovery time. This conversion of qualitative data into a quantitative format directly compares freezing rates, thawing rates, and recovery times across multiple experimental replicates.

By implementing this quantitative framework, statistically robust comparisons can be performed across the tardigrade population, yielding valuable insights into their remarkable ability to withstand freezing and subsequently recover. These quantitative analyses will not only enhance our understanding of tardigrade cryotolerance but also provide a foundation for future investigations into the underlying mechanisms that enable this extraordinary biological phenomenon.

Future work:

Future work will focus on developing a user-friendly application based on the current code, thus broadening access for researchers seeking to translate qualitative observations into comparable quantitative data. Furthermore, the potential applicability of this application to other research areas characterized by qualitative data requiring quantification and inter-study comparison will be investigated to facilitate the identification of significant patterns and the generation of new insights across diverse scientific disciplines.

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Supplementary material:

https://colab.research.google.com/drive/1ihrHIK1BpCfGga9tUYxPt72K6E-eiW8l?authuser=1#scrollTo=WJzx-qxSxGCL