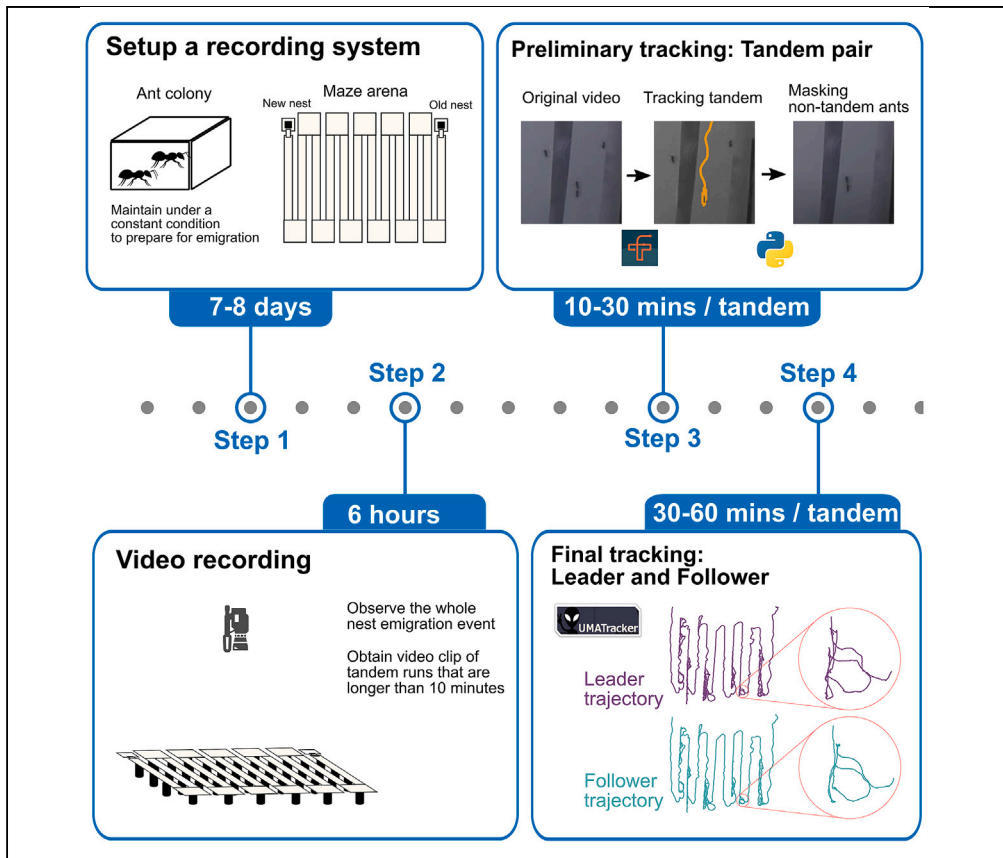


## Protocol

# Protocol to obtain long movement trajectories of leaders and followers in ant tandem runs



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### Highlights

Record long ant tandem runs with a single camera in the experimental arena

Integrative tracking extracts coordinates of focal tandem pairs among other ants

Long tandem trajectories are used to infer stability and direction of interactions

The enhanced efficiency of tracking aids comparative behavioral analysis across species

Tandem running in ants is a sophisticated form of communication. Precise measurement of movement coordination by the tandem pair can shed light on social interactions. Here, we describe an integrative approach to obtain long movement trajectories of a specific tandem pair within a crowd of ants. We describe a maze-like arena, video recording and editing, and movement tracking. We integrate two pieces of image-based tracking software that have distinct individual assignment strategies. This protocol aids comparative studies of recruitment communication across species.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Protocol

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## SUMMARY

Tandem running in ants is a sophisticated form of communication. Precise measurement of movement coordination by the tandem pair can shed light on social interactions. Here, we describe an integrative approach to obtain long movement trajectories of a specific tandem pair within a crowd of ants. We describe a maze-like arena, video recording and editing, and movement tracking. We integrate two pieces of image-based tracking software that have distinct individual assignment strategies. This protocol aids comparative studies of recruitment communication across species.

For complete details on the use and execution of this protocol, please refer to Mizumoto et al. (2023)<sup>1</sup> and Valentini et al. (2020).<sup>2</sup>

## BEFORE YOU BEGIN

The protocol described below has been used to investigate leader-follower interaction during tandem runs in *Diacamma cf. indicum* by quantifying information flow between partners. Our approach builds upon the methods described in a previous study of tandem running in *Temnothorax rugatulus*,<sup>2</sup> enhancing the efficiency of data acquisition beyond what was originally proposed. By applying this protocol to other ant species (e.g., Schultheiss et al., Silva et al., and Gruter et al.<sup>3–5</sup>), we can better understand the origins and evolution of tandem communication, which has independently arisen 15 times among the ants.<sup>1,6,7</sup> Our protocol focuses on tandem running during colony emigration, but it can also be applied to tandem recruitment during foraging by substituting food items in place of the new nest. Furthermore, this protocol can be used to obtain trajectories for other ant recruitment mechanisms that involve a relatively small number of individuals, e.g., group recruitment in several species of *Camponotus*. The size of the experimental arena may need to be adjusted when testing ant species other than *Diacamma cf. indicum*.

## Preparing ant colonies

⌚ Timing: 8 days



1. Collect ant colonies and bring them to your laboratory.

**Note:** Colonies of *Diacamma cf. indicum* nest underground. After identifying a nest entrance, we excavated the nest with a shovel and collected all individuals using a vacuum.

**Note:** In this species, a mated worker with the queen's function is called a "gamergate." We use the term "queen" instead of gamergate in this paper.

△ **CRITICAL:** If needed in your region or collection site, obtain the relevant permits for scientific collection and importing/exporting of living ant colonies. The requirements and regulations for obtaining a permit vary between localities and countries.

△ **CRITICAL:** Make sure that the colonies contain the queen(s). Lack of queen(s) causes physiological and behavioral changes in workers (e.g., Peeters and Tsuji and Shimoji et al.<sup>8,9</sup>) and thus may elicit unusual nest emigration events.

2. Introduce a colony into a humid artificial nest covered by a red plastic plate connected to a foraging area. We used a plastic container (148 × 84 × 37 mm) half-filled with moistened plaster as an artificial nest and put it into a large container that serves as a foraging area (240 × 180 × 85 mm). As ants prefer a humid and dark place as a nest, they immediately move into the artificial nest after they are released in the foraging arena, whose walls are coated with Fluon to prevent ants from escaping.
3. Maintain colonies until the beginning of the experiment. Water daily and provide mealworms or a standard artificial diet<sup>10</sup> once every 3 days.

**Note:** We maintained colonies for at least 7 days in the laboratory (14L:10D cycle and 24 ± 1°C). During this period, we confirmed if the colony condition was normal and suitable for nest emigration experiments (e.g., no mass mortality after introduction to the new nest, no death of the queen due to disturbance, and eating food regularly).

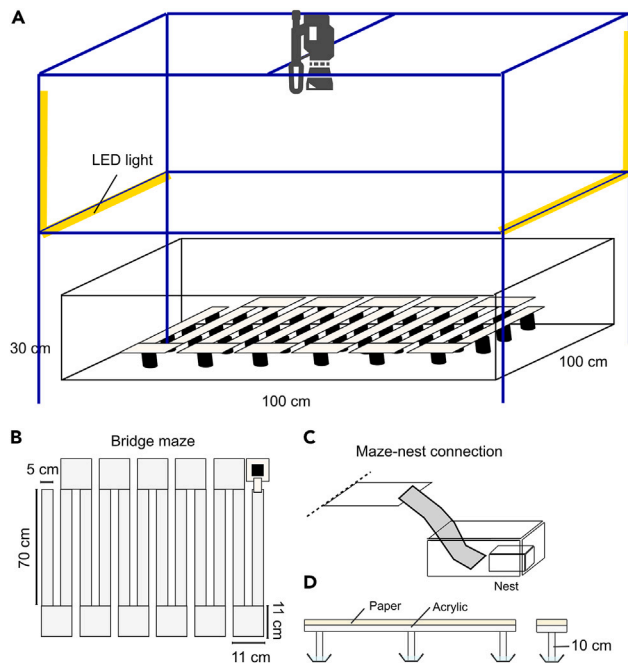
**Note:** Maintenance conditions, including food and design of artificial nest, should be adjusted to the studied ant species.

### Setting up maze arena

⌚ **Timing:** 1 day

Colony emigration by ants is observed in a maze arena, designed to make the ants travel long distances within a confined space. This allows the observation of long tandem runs within a space small enough to be captured by a single camera. Here we introduce a maze made of bridges. See [limitations](#) for a possible alternative solution.

4. Prepare an experimental maze to get a sufficient distance between the old and new nests ([Figure 1A](#)).
  - a. Prepare 12 straight boards (5 × 70 cm) for maze arms and ten square boards (11 × 11 cm) for U-turns to connect the arms to each other. Boards are made of acrylic plates (2 mm thickness) covered with white cardboard.
  - b. To elevate the maze above the arena floor, attach three poles (10 cm high) to the bottom of each straight board, and a single pole to the bottom of each square board, using a glue gun ([Figure 1D](#)).
  - c. Attach the other side of each pole to a plastic cup (13 × 6 cm) using a glue gun.
  - d. Assemble the straight and square boards to form a maze ([Figure 1B](#)).
  - e. Fill each cup under the pole with water.
  - f. Place the maze arena inside an acrylic container (100 × 100 × 30 cm) without a lid.
  - g. Apply Fluon to the walls of the container.



**Figure 1. Experimental maze arena to obtain long tandem trajectories**

- (A) Overview of the experimental setup. Blue lines indicate the frame for LED lights and the video camera.  
 (B) Overhead view of the bridge maze, showing its size.  
 (C) Close view of the maze-nest connection.  
 (D) Side view of the bridge maze showing pole supports in water-filled cups to prevent ants leaving the maze.

△ **CRITICAL:** Confirm that the Fluon and water cup can prevent ants from escaping from the arena. In case ants overcome these barriers, other countermeasures will be required.

**Note:** The size and shape of the arena should be adjusted depending on the ant species. In this protocol, we aim to obtain trajectories of tandem runs longer than 10 min. This is estimated to be sufficient for inferring information transfer between leaders and followers, based on a power analysis on *Temnothorax rugatulus* and two termite species.<sup>2</sup> Note that the minimum duration might change from species to species, and longer trajectories are generally preferable when possible. As a rule of thumb, a total distance of about 1,000 body lengths is sufficient. This may be because average moving speed of ants was less than 1.5 body lengths /s (it will take >10 min to move 1,000 body lengths) in our analyzed pairs.<sup>1,2</sup> For example, in *Diacamma cf. indicum* (body length ~10.5 mm), ants had to travel at least 9,500 mm within the maze to reach a new nest. In a previous study with *Temnothorax rugatulus* (body length ~2.3 mm), ants had to travel 2,520 mm within a maze arena, which was made by dividing a flat surface by walls rather than bridges.

**Note:** Ants can go to the underside of the bridge and thus out of the camera's view. However, at least in *D. cf. indicum*, they prefer to walk on the top side of the boards (ants went under the bridge only ~2.20% of the time<sup>1</sup>). This problem might be solved by applying Fluon under the bridge or using a planar arena with a maze constructed of thin walls (see also Limitations).

- h. Prepare a steel frame (120 × 120 × 200 cm) around the arena, attach four LED lights to the frame (100 cm high from the bottom), and set a digital video camera above the arena (Figure 1A). The video camera has to be oriented perpendicular to the plane of the bridge to avoid scaling differences across the area of the video. Cover the entire frame with blackout curtains to create a dark room.

△ **CRITICAL:** High temperature could affect ant behavior, and thus, lighting sources that produce excessive heat (e.g., incandescent light bulbs and fluorescent lamps) should be avoided. LED lights are preferred as they usually emit less heat. The behavioral observations in *Diacamma* ants were performed under  $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$  and 40% humidity.

- i. Prepare two plastic containers (240 × 180 × 85 mm, with walls coated by Fluon) as a space to introduce ant nests. These containers are connected to the bridge maze with a piece of paper (Figure 1C).

**Note:** We used a Panasonic video camera (HC-VZX992M) with 1 K resolution (1920 × 1080) and 60 FPS. With this resolution setup, individual *Diacamma* ants were about 9 pixels, which is sufficiently big for automatic tracking analysis.

**Note:** In this protocol, the aspect ratio of the arena was 1:1. However, ideally, this should be the same as the aspect ratio of the video recording (16:9 in this case) to enable the camera to capture the entire arena using the full video frame and thus make it easier to obtain longer maze paths.

△ **CRITICAL:** Any video camera can be used as long as it can cover the whole arena with a reasonable size of ants (e.g., 9 pixels). However, video cameras suitable for long-term recording are preferable (e.g., Panasonic HC-X2000). Our previous choice of Panasonic HC-VZX992M was not optimal because it automatically produces a short blackout between successive video clips. In the previous study, we interpolated the coordinates of ants between the last frame and the first frame before and after the blackout period.<sup>1</sup> Also, be aware that using a camera with a large field of view (FOV) (e.g., GoPro) causes a scale issue, where objects near the edge of the frame appear smaller than they really are. Video cameras with a close to orthographic FOV over the bridge area are preferable.

△ **CRITICAL:** Make sure that the camera is oriented perpendicular to the plane of the bridge to avoid distortion of the recording frames.

### Installation of required software and packages

⌚ **Timing:** 10 min

5. Install Python (Python  $\geq 3.8$ ) on your computer system or in a virtual environment. For new users of Python, we recommend using Anaconda. Here we describe the protocol using Anaconda.
6. Download and install Anaconda for your operating system from <https://www.anaconda.com/>.
7. Install FastTrack from <https://www.fasttrack.sh/docs/interactiveTracking/> and UMATracker from <https://ymnk13.github.io/UMATracker/>.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
<i>Diacamma cf. indicum</i>	Kwansei Gakuin University	Collected in Okinawa, Japan
Software and algorithms		
FastTrack	Gallois and Candelier (2021) <sup>11</sup>	<a href="https://doi.org/10.1371/journal.pcbi.1008697">https://doi.org/10.1371/journal.pcbi.1008697</a>
UMATracker	Yamanaka and Takeuchi (2018) <sup>12</sup>	<a href="https://doi.org/10.1242/jeb.182469">https://doi.org/10.1242/jeb.182469</a>
Python	<a href="https://www.python.org/downloads/">https://www.python.org/downloads/</a>	RRID: SCR_008394

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
R Project for Statistical Computing	R Core Team	RRID_SCR_001905
QuickTime player	Apple	
Other		
iMac	Macintosh	
Digital video camera	Panasonic	HC-VZX992M
LED light	IRIS OHYAMA	IBA9D-W

## STEP-BY-STEP METHOD DETAILS

### Video recording of ant nest relocation

⌚ Timing: 6 h

This section describes the protocol for recording nest emigration in ants.

1. Introduce the ant colony to the maze arena by placing their current nest at the beginning of the maze. Also, place an empty artificial nest at the other end of the maze.
2. Open the upper lid of the current nest to promote nest relocation and start recording.
3. The recording will last until all individuals move to the new nest.

**Note:** Nest relocation is stimulated by opening the lid of the container, which is a practice widely used in ant nest emigration experiments, e.g., Mukhopadhyay and Sumana, Pratt et al., and Pratt.<sup>13–15</sup> Following nest destruction, ants wander around the maze arena to look for a potential nest site. Once they find the new nest and begin to recruit to it, colony emigration begins. In *Diacamma cf. indicum*, all individuals, including queens, move from the old nest to the new nest via tandem runs. Nest emigration is completed when all individuals move into the new nest. Including exploration and emigration, it requires up to 6 h for *Diacamma cf. indicum* to complete an emigration in this setup. It may take different periods for different ant species.

⚠ **CRITICAL:** Emigration videos will be analyzed by tracking software that uses image binarization as an algorithm to detect individuals. Thus, consistent lighting during recording is essential. Make sure that there are no light fluctuations during recording due to, e.g., observer's shade, sunset, sunrise, and changes in other light sources. For example, the experimental setup can be in an isolated dark room to minimize disturbance. Also, turn off your camera's automatic brightness adjustment function and use manual focus to minimize unintended fluctuations of the images.

⚠ **CRITICAL:** Similarly, human presence near the experimental setup may affect the ants' movements. The experimental setup should be isolated during nest relocation.

### Choosing the tandem events to analyze

⌚ Timing: 3–4 h (per colony)

In this step, we describe the protocol to preprocess the recorded videos for further analysis. During nest relocation, most individual ants are led in tandem runs, hence the total number of runs can be very large. However, we do not analyze all these events. Instead, we focus on a subset of tandem runs that took at least 10 min to be completed. These longer runs provide enough data for the information theory analysis described in Valentini et al.<sup>2</sup> and Mizumoto et al.<sup>1</sup> The original videos are subdivided into several clips, each of which includes a focal tandem running event.

4. Measure the length of tandem running events using the focal sampling method. Repeat this for all tandem runs to obtain enough samples of long runs.
  - a. Open videos with a media player (e.g., QuickTime Player 10.5) and locate the frame at which the tandem run initiates at the old nest. Note the time of this frame so that you can later measure how long this tandem run has lasted.

**Note:** Any video editing software can be used as long as it has a trimming function (e.g., PowerDirector 365 and Final Cut Pro).

**Note:** Sometimes, a tandem run can start in the middle of the arena due to encounters between lost or solo individuals. In this protocol, we only focus on tandem runs that start at the old nest and end at the new nest.

- b. By playing videos and moving the seek bar, follow the movement and outcome of the focal tandem run.
- c. If the focal tandem run breaks up and one of the partners starts tandem running with another ant, stop the observation and go back to 4a for the next tandem run.

**Note:** Tandem runs sometimes temporarily break up when the partners separate a short distance before later reuniting. This separation-reunion process is an integral part of analyzing social interactions. In most cases, ants reunite within a short period (about several seconds). However, when separated, individuals may encounter and pair with other tandems/solo individuals/other separated individuals. Such events are not suitable for studying the leader-follower relationship of specific partners. These events happen when there are many tandem pairs on a bridge, especially in a large colony.

**△ CRITICAL:** Multiple tandem pairs can be found at the same time in the maze arena. Especially for the frame in which the focal tandem pair movement intersects with other tandem pairs, careful observation is required not to confuse tandem pairs with each other. Speed control of video playback can enhance the efficiency of observation.

- d. If the focal tandem run arrives at the new nest, stop the observation and note the time of the frame at which the tandem arrived. By referring to the initial frame recorded at 4a, check if the tandem lasted more than 10 min. If it was less than 10 min, reject this tandem and return to 4a to find the next tandem run.
- e. If the focal tandem duration is at least 10 min, save a video clip extending from the initial to the final frame of the tandem run.
- f. Go back to 4a for the next tandem run.

**Note:** We collected the first five tandem running events that exceeded 10 min for each colony emigration.

### Preliminary tracking of tandem runs as a pair

⌚ Timing: 10–30 min (per tandem)

After obtaining video clips, the following sections describe detailed steps for movement tracking. Movement tracking consists of three different processes. First, among many ants in the experimental arena, find the focal tandem pair and extract the coordinates of the center of the pair. For this purpose, we use FastTrack software<sup>11</sup> as this allows a variable number of individuals to be present in the video image. Other software with similar specifications may be compatible.

However, any software that requires a fixed number of individuals to be present in the video image cannot be used.

5. Start FastTrack and open the video file to analyze.
  6. Specify the image options.
    - a. Create a background using the Average method with 100 images.
    - b. Set the region of interest (ROI) to only include the experimental arena.
    - c. Binarize the image with the light background option to detect tandem pairs. The appropriate threshold will depend on the recording conditions. We used values around 4–16 for the example analysis.
    - d. Perform morphological operations on binary images to improve image segmentation of tandem running pairs. Ideally, tandem pairs should be represented by a single object. We use the Dilation operation with the Rectangle Kernel (size = 1–8) to smooth the boundaries between leaders and followers.
    - e. Set the “Detection” parameter to detect tandem pairs but exclude solo ants. Thus the minimum size should be larger than single ants, and the maximum size should be slightly larger than tandem pairs. We use the values 45–80 and 300–500, respectively.
  7. Specify Tracking options.
    - a. Tune tracking parameters with the option Spot to track: Body. We use the Maximum Time (T) = 60–100 and Maximum Distance (L) = 30–60. Other parameters are automatically determined by clicking the “Level” button.
    - b. Perform Preview to check if tracking is performed as intended.
    - c. Run Track. It will take 10–30 min, depending on your computers’ specifications and video length/resolution.
- Note:** As FastTrack detects the tandem pair as a single object, it fails to detect pairs when the partners are separated. When the pair was not detected for a while and then detected again due to separation-reunion processes, two parameters, Maximum Time (T) and Maximum Distance (L), are used to determine if these objects are identical. If the object was not detected for less than T and moved less than L during this period, the identity of the object is held. These two parameters may need to be adjusted depending on the species.
8. Manually correct the tracking errors using Tracking Inspector.
    - a. Name the focal tandem pair as “0” and go through the video. Assign “0” to the focal tandem pair if the tandem identity was swapped.
    - b. The data will be autosaved. The results are in the directory “Tracking\_Result\_(Filename).” The “Export” button will generate a video with annotations.
    - c. Obtain finalized tracking.txt in the result directory.

### Creating videos containing only the focal tandem

⌚ Timing: 15–30 min (per tandem)

Using the coordinates of the center of the tandem running pair obtained above, this step generates videos that will be used for steps 15–18: *Final tracking of the leader and the follower*. Because FastTrack skips any frame in which it fails to recognize an object, coordinates in skipped frames are assigned by calculating the averaged values of the frames before and after the skipped frames. Then, a video that shows only the focal tandem running pair is generated. This is accomplished by using a background image to mask all regions outside the area immediately surrounding the coordinates of the tandem pair. The Python script that performs all these processes is at [http://www.github.com/nobuaki-mzmt/ant\\_tandem\\_mask](http://www.github.com/nobuaki-mzmt/ant_tandem_mask) (Python  $\geq$  3.8).



9. Place the original video files and the FastTrack .txt result files in the same folder. These two should have the same name but different extension, e.g., Ant-video-01.mp4 and Ant-video-01.txt.
10. Open an Anaconda Prompt (Anaconda 3).
11. Download the Python code GenerateMaskVideo.py from the GitHub repository [https://github.com/nobuaki-mzmt/ant\\_tandem\\_mask](https://github.com/nobuaki-mzmt/ant_tandem_mask). This can be done in two ways:
  - a. Click in the 'Code' tab, download the zip folder, and extract all files.
  - b. Alternatively, run the following command line if the *git* tool is installed on the computer.

```
> git clone https://github.com/nobuaki-mzmt/ant_tandem_mask.git
```

12. Run GenerateMaskVideo.py from Anaconda Prompt.

```
>cd [name-of-folder-that-includes-GenerateMaskVideo.py]
>python GenerateMaskVideo.py
```

13. The GUI window will automatically pop up to specify the folder created at step 9 (Figure 2).

**Note:** It will also ask for the "Tandem window size," which is 20 pixels by default. This parameter specifies a square region around the tandem pair coordinates that is not masked by the background image.

**△ CRITICAL:** The best value for this tandem window size depends on the recording setup and potentially on the species' behavior. Larger values will be required if the recorded individuals fill a larger pixel space and when a long distance often separates tandem leaders and followers (Figure 2C). However, at the same time, a larger window size will tend to include other individuals in the non-masked area. See also Notes in the following section (Figure 2B).

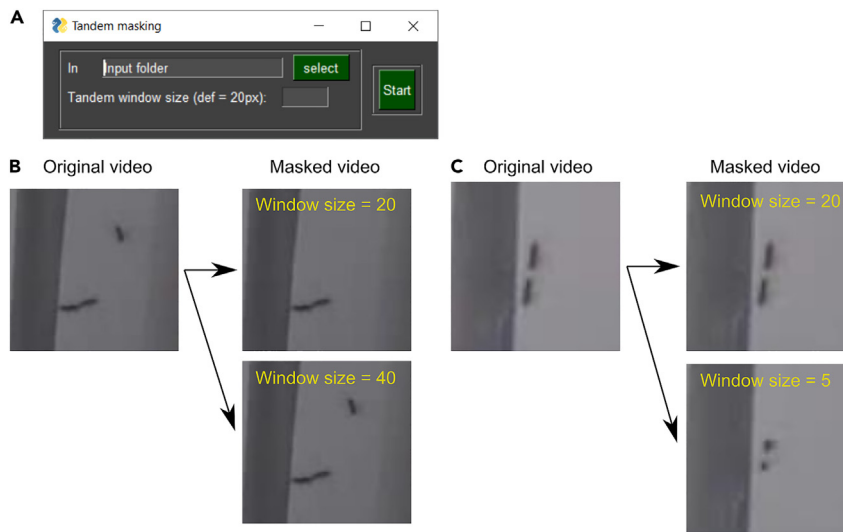
14. Generated videos will be automatically stored in the output folder.

### Final tracking of the leader and the follower

⌚ Timing: 30–60 min (per tandem)

This section describes the second stage of tracking to get detailed tracks of both leaders and followers from the masked video generated at step 14. We recommend using UMATracker software because it usually performs well with tandem runs in ants<sup>1,2</sup> and termites.<sup>16,17</sup> If UMATracker is unavailable, try other software that assumes a fixed number of individuals.

15. UMATracker consists of four programs: UMATracker-FilterGenerator; UMATracker-Tracking; UMATracker-TrackingCorrector; UMATracker-Area 51.
16. Execute UMATracker-FilterGenerator and load the video file.
  - a. Select Background / Create background and perform background removal.
  - b. Binarize the video. An example of the combination of Filter Block Operation is "output <- Threshold (30–45) <- BGRTtoGray <- Rectangle Selection <- input". Save the filter after confirming that ants are shown as white blobs and other regions are in black.
17. Execute UMATracker-Tracking and drag and drop the video file and filter file.
  - a. Set the parameter of # of objects = 2, indicating the leader as 0 and the follower as 1.
  - b. Run the object tracking.



**Figure 2. The relationship between “Tandem window size” and the output masked video**

(A) The pop-up GUI window.

(B) Larger windows tend to include other ants.

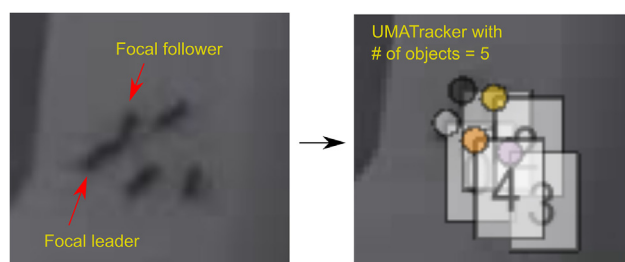
(C) With smaller window sizes, tandem partners may be cut off.

- c. After the tracking is finished, select Save Data to save a CSV file with ant coordinates across frames.
- d. If the # of objects is set > 2, open the CSV file and delete the columns for the objects other than the leader and the follower.

**Note:** If many ants come in and out of the frame, it may be easier to set the number of objects > 2 (Figure 3). When the number of objects is set >2, but there are only two ants in the frame, UMATracker assigns two points to a single individual. Make sure 0 is always on the leader while 1 is always on the follower.

18. Execute the Tracking Corrector and drag and drop the video and CSV files.
  - a. Move the seek bar to confirm that the leader has identifier 0 and the follower has identity 1 in all frames.
  - b. If there is a swapping of identity between leader and follower, right-click the markers to correct the error.

**△ CRITICAL:** In frames where the leader and the follower are separated by more than the threshold distance (indicated at “Tandem window size” in the previous Python script; see step 13), ants may not be detected in the masked video frames. For these frames,



**Figure 3. An example in which high local ant density has led to more than two ants appearing in the frame of the masked video**

**Table 1. Head of raw data output from movement tracking by UMATracker**

Position	x0	y0	x1	y1
0	1079.711	18.22003	1081.997	11.01635
1	1079.618	18.34953	1081.724	11.56355
2	1079.786	18.99798	1081.856	12.15008
3	1079.878	19.19997	1081.818	12.37936
4	1079.848	19.75074	1081.805	12.39247
5	1079.667	20.58345	1081.714	12.93159
6	1079.715	21.35219	1081.715	13.35557
7	1079.714	21.78718	1081.713	13.93255
8	1079.715	22.2119	1081.5	14.64491
9	1079.714	22.2172	1081.179	15.10594
10	1079.187	23.18751	1081.364	15.2732
11	1079.266	23.40311	1080.914	15.92748
12	1079.201	23.73027	1080.915	16.25846
13	1079.267	24.19977	1080.665	17.08977
14	1079.125	24.62664	1080.665	17.09048
15	1079.231	25.61689	1080.332	17.92122
16	1079.199	26.07133	1080.499	17.92709
17	1079.385	26.69221	1080.454	18.2746
18	1079.384	26.54122	1080.499	19.00867
19	1079.399	26.93541	1080.454	19.2876
20	1079.312	27.49924	1080.5	19.50236
21	1079.188	27.49108	1080.455	20.2767

Column information: (1) frame number, which is equivalent to seconds when divided by FPS, (2) x coordinate of the leader, (3) y coordinate of the leader, (4) x coordinate of the follower, and (5) y coordinate of the follower.

it may be easier to do another tracking using the original video by selecting ROI larger than "Tandem window size." When ants other than the focal tandem pair are detected in this ROI, set the parameter "# of objects" as larger than the maximum number of ants in this region. When the number of ants in the frame is smaller than the "# of objects," UMATracker assigns multiple points to a single individual (see the note above too). Again, ensure 0 is always on the leader while 1 is always on the follower. This separate tracking does not need to cover all the video frames. Instead, this focuses on obtaining the coordinates of the leader and follower only in the frames where ants are not detected in the masked video. UMATracker needs to start tracking from the beginning of the video, but the software may be stopped once it has completed tracking the frames of interest. Then, open the CSV file, copy the coordinates of the leader and follower in the corresponding frames, and manually merge these coordinates into the main CSV file. The CSV files look like [Table 1](#), and the "position" row indicates the frame number. Make sure to match the frame numbers of the main CSV file and the CSV file of the separate tracking.

## EXPECTED OUTCOMES

CSV files containing tandem leader and follower coordinates for each frame are obtained. The number of frames depends on the frames per second (FPS) and the video length in your recording setup. With 30 FPS and 7 min 4 s tandem video, we obtain a data table with 5 columns and 25704 rows ([Table 1](#)).

## QUANTIFICATION AND STATISTICAL ANALYSIS

This protocol aims to obtain long trajectories for ant tandem runs. The detailed description of how these trajectories are subsequently analyzed is beyond the scope of this protocol. However, to highlight the advantage of obtaining long trajectory datasets of tandem runs, here

we summarize the analysis of coordination data from UMATracker as conducted in previous studies with ants and termites.<sup>1,2,18</sup> The data are analyzed in R scripts. The source code is available in those papers.

### Scaling the datasets

The unit of coordinate values in datasets obtained from UMATracker is the pixel. For analyses across different videos, the value should be scaled into mm, a unit of length in the International System of Units (SI). Also, the value will be divided by the body length of individuals (mm) for cross-species comparison.

Measure the length of known objects (such as the experimental arena) in pixels.

1. Get a screenshot image of the video frame.
2. Open the image with basic image analyzing/editing software, such as ImageJ, Inkscape, or Illustrator, and measure the length of the long side of the arena (you will get smaller measurement errors with the long side than with the short side.). Or, measure the length of both sides of the arena, corresponding to x and y directions of the frame, to calculate scales for x and y axes separately.

**Note:** Additional video calibration may be required depending on camera setups, e.g., unusual FOV. Here are some useful links for the calibration; OpenCV with Python: [https://docs.opencv.org/4.x/dc/dbb/tutorial\\_py\\_calibration.html](https://docs.opencv.org/4.x/dc/dbb/tutorial_py_calibration.html). Camera calibrator in MATLAB: <https://jp.mathworks.com/help/vision/ref/cameracalibrator-app.html>.

3. Calculate scale = (Object length in mm) / (Object length in pixels).
4. Multiply all the coordinate values of datasets by the value of scale.

### Stability of tandem runs

One advantage of obtaining long tandem runs is to evaluate how stable tandem runs are for the species, which can be an indicator of the nature of the leader-follower relationship.<sup>1,18,16</sup> Ants in tandem running pairs often separate spontaneously and then reunite after a short period of time. Thus, such stability can be quantified by the duration of tandem runs: how long each tandem pairing lasts without interruption.

5. For each frame, determine if the leader and the follower are in tandem. Pairs can be regarded as engaging in tandem runs if the distance between their centroid is less than two body lengths.<sup>2</sup>

```
>## Table 1 is read as df
>df$Distance <- sqrt( (df$x0 -df$x1)^2 + (df$y0 -df$y1)^2 )
>tandem_state <- df$Distance < 2 * body_length
```

6. Calculate the duration of each tandem running event:

```
>tan.start = which(tandem_state) [c(T, diff(which(tandem_state))>1)]
>tan.end = which(tandem_state) [c(diff(which(tandem_state))>1, T)]
>frame.zero = which(df$position == 0)
>frame.end = which(df$position == max(df$position))
```

```

>tan.start = unique(sort(c(tan.start, frame.zero)))
>tan.end = unique(sort(c(tan.end, frame.end)))
>tandem.duration = tan.end - tan.start + 1
>tandem.censor = tan.end %in% frame.end
  
```

7. "tandem.duration" is a vector for the duration of each tandem running behavior. The observation of the last tandem running event must be censored due to the end of the video. This information is stored in "tandem.censor".
8. The outcome can be analyzed using survival time analysis, using R packages, such as "survival," "survminer," and "coxme."

### Information transfer

Another advantage of obtaining long tandem runs is to be able to perform information-theoretical analysis. Previous studies revealed that the net direction of information transfer between leaders and followers could be qualitatively and quantitatively distinct between different ant and termite species.<sup>1,2,18</sup> Such patterns were quantified by computing transfer entropy using the 'rinform-1.0.1' package in R.<sup>19</sup> Refer to Valentini et al.<sup>2</sup> for a detailed explanation of the underlying idea and its background idea as well as full R scripts. Here some core parts of the scripts are introduced as an example of quantifying information transfer.

9. Discretize trajectories of leaders and followers. Two types of time series are obtained: pausing pattern and rotation pattern.
  - a. The pausing pattern has two states, pause and move. This can be obtained using a move/pause threshold for displacement between two successive frames as follows:

```

># for the coordinate vector X and Y of an ant,
># obtain Pausing pattern vector trackM
>encodeMotion <- function(x, y, threshold) {
> X <- matrix(c(x, y), 2, 2)
> d <- dist(X)
> if (d <= threshold) {
> # No motion state: 0
> entry <- 0
> } else {
> # Motion state: 1
> entry <- 1
> }
> entry
>}
>
>X <- df$x0
>Y <- df$y0
>trackM_leader <- rep(0, length(X))
  
```

```
>for (t in 2:length(X)) {  
> trackM_leader [t] = encodeMotion(X[(t-1):t], Y[(t-1):t], threshold)  
>}
```

**Note:** The threshold was set as the 10th percentile of the probability distribution of step length after inspection of the corresponding probability distribution.<sup>2</sup>

- b. The rotation pattern has two states, clockwise and counterclockwise rotation. These two states are distinguished based on the direction of motion computed as the cross-product of movement vectors between three successive frames. If no rotation was detected (i.e., cross-product equal to 0), the direction is copied from the previous time step.

```
# for the coordinate vector X and Y of an ant,  
# obtain Rotation pattern vector trackR  
>encodeRotation <- function(x, y, previous) {  
> v1 <- c(x[2] - x[1], y[2] - y[1])  
> v2 <- c(x[3] - x[2], y[3] - y[2])  
> rotation <- v1[2] * v2[1] - v1[1] * v2[2]  
>  
> if (rotation < 0.0) {  
> # Turn clockwise state: 0  
> entry <- 0  
> } else if (rotation > 0.0) {  
> # Turn counter-clockwise state: 1  
> entry <- 1  
> } else if (rotation == 0) {  
> # Collinear, no rotation: use previous entry if valid or  
> # random rotation if not  
> if (previous != -1) {  
> entry <- previous  
> } else {  
> entry <- rbinom(1, 1, 0.5)  
> }  
> }  
> entry  
> }  
> trackR_leader <- rep(0, length(X))  
>for (t in 2:(length(X)-1)) {  
> trackR_leader[t] = encodeRotation(X[(t-1):(t+1)], Y[(t-1):(t+1)], >trackR_leader [t-1])  
>}
```

10. After obtaining discretized time-series datasets, compute transfer entropy between a leader and a follower for both directions: from leader to follower and from follower to leader.

```
># the discrete time-series vector for a leader: trackM_leader, and a follower:
trackM_follower

>library(rinform)

>TElf <- mean(transfer_entropy(trackM_leader, trackM_follower, k = 2, local = T)) # leader ->
follower

>TEfl <- mean(transfer_entropy(trackM_follower, trackM_leader, k = 2, local = T)) # follower
-> leader
```

**Note:** The parameter  $k$  is the history length of transfer entropy. In previous studies,  $k$  is explored in the range from 1 to 20 to find the parameter value that maximizes information transfer.

**Note:** Another parameter is the sampling frequency. The original videos, and thus the UMATracker tracking results, are in 60 FPS. We changed this sampling frequency by down-sampling the tracking results in the range from 30 FPS to 0.75 FPS to find the parameter value that maximizes information transfer.

11. Compare the strength of information flow between two directions: from leader to follower and from follower to leader.

```
># After calculating TElf and TEfl for all individuals

>wilcox.test(TElf_all, TEfl_all, alternative = "greater", paired = T)

>wilcox.test(TEfl_all, TElf_all, alternative = "greater", paired = T)
```

## LIMITATIONS

First, although our protocol enables semi-automatic tracking of tandem running pairs in the presence of other ants, the effectiveness is only validated in *Diacamma cf. indicum* so far. We suppose that our protocol is also suitable for *Temnothorax rugatulus* as similar solutions were employed in the previous study.<sup>2</sup> In either case, the effectiveness depends on the density of ants surrounding tandem running pairs. For example, when the tandem pairs go through narrow parts of the maze and the density of ants is high, a more manual process will be required. Also, manual correction is necessary when partners are separated at a distance. Therefore, analyzing tandem runs of ant species with more spontaneous separation is expected to take more time due to manual corrections.

Second, our bridge system could be improved. Because the maze-shaped bridge inevitably includes many turns and narrow paths, many ants sometimes get stuck in these places. In such a high-density area, contacts between ants cause the interruption of tandem running pairs. Reducing the turning angles of the U-turns or widening the bridges could solve this problem by helping ants move more smoothly. Furthermore, ants can move to the back side of the maze, which happens for focal tandem pairs. Therefore, it is necessary to carefully check that the tandem pair has reached the new nest without being replaced by another leader or follower.

Alternatively, a maze arena could be created by dividing a large planar arena with thin walls as in a previous study.<sup>2</sup> This system could be more suitable for small and less active ant species, such as *Temnothorax*, because they require relatively low walls that have small blind spots from the cameras. On the other hand, the wall-dividing system may not be a good solution for large and active ant species. For

example, workers of *Diacamma cf. indicum* often succeed in climbing up the wall even with Fluon coating. Also, the larger body size of *Diacamma cf. indicum* requires taller walls that have larger blind spots from cameras. Blind spots can be reduced by locating the camera at an elevated position and increasing the distance between the camera and the arena. However, this is not always possible in lab environments.

### TROUBLESHOOTING

#### Problem 1

Ants escape from the maze (related to Step 4 in the 'Set up maze arena' Step 4).

#### Potential solution

- Confirm that the paper bridge connecting the old nest and the maze does not touch the edge of the outer container. Usually, *Diacamma* ants do not escape as long as the bridge is separated from the outer container by more than 2 cm.
- Also, ensure that all water cups under the poles are filled with enough water. If using species that can swim, consider using oil instead of water.

#### Problem 2

No tandem running events are longer than 10 min (related to Step 4 in the 'Choosing the tandem events to analyze' Step 4).

#### Potential solution

- The experimental arena is too small given the ant body size and moving speed. Add more bridges to ensure that ants need to move long enough distance for nest emigrations.

#### Problem 3

The ants' bodies are cut off (or wholly missing) from the video generated from `GenerateMaskVideo.py` (related to Step 11 in section on Creating videos containing only focal tandem).

#### Potential solution

- First, ensure that the focal tandem pair is named "0" in FastTrack and tracked accurately throughout the clip.
- If the focal tandem pair is separated far enough that the partners end up in the masking area, increase the "tandem window size" in `GenerateMaskVideo.py` to include the separated partners.
- If only a small portion of the frames is missing, it may be easier to track from the original video (See Note in Second tracking of the leader and the follower).

#### Problem 4

Other ants appear in a video generated from `GenerateMaskVideo.py` (related to Step 11 in section on Creating videos containing only focal tandem).

#### Potential solution

- Reducing the "tandem window size" parameter in `GenerateMaskVideo.py` makes it possible to reduce the number of non-focal ants in the video. However, if only a portion of the frames contain other ants, it may be easier to change the number of objects tracked by UMATracker (see note on Second tracking for leader and follower).

#### Problem 5

Individual ants are not appropriately tracked with UMATracker (related to Step 15 in the section on [final tracking of the leader and the follower](#)).



### Potential solution

- Ensure that the filter shows leader and follower ants as white blobs. Decreasing the “threshold” may help solve this problem.
- Ensure there are no small blobs other than focal ants in the filter. Increasing the “threshold” or adding the “Morphology” operation will reduce noise in the video.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Nobuaki Mizumoto ([nobuaki.mzmt@gmail.com](mailto:nobuaki.mzmt@gmail.com)).

#### Technical contact

The technical contact for this protocol is Hiroyuki Shimoji ([toireikitaimitai@gmail.com](mailto:toireikitaimitai@gmail.com)).

#### Materials availability

The study did not generate new unique reagents.

#### Data and code availability

The code generated during this study is available at GitHub: [https://github.com/nobuaki-mzmt/ant\\_tandem\\_mask](https://github.com/nobuaki-mzmt/ant_tandem_mask). The accepted version is available at Zenodo: <https://doi.org/10.5281/zenodo.10127100> as a version of record.

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### AUTHOR CONTRIBUTIONS

Y.T., conceptualization, methodology, formal analysis, investigation, resources, data curation, writing – original draft, and writing – review and editing. G.V., conceptualization, methodology, software, resources, and writing – review and editing. S.C.P., conceptualization, methodology, writing – review and editing, and supervision. H.S., conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review and editing, supervision, project administration, and funding acquisition. N.M., conceptualization, methodology, software, validation, formal analysis, data curation, writing – original draft, writing – review and editing, visualization, supervision, project administration, and funding acquisition.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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