Illustrated Protocol for Processing Radiometric Videos Using the Software *FLIR Tools :*

An Approach Applied to a Novel Methodology for Lizard Thermal Exchange Rates Studies

Frederico M. Barroso*[,a,b,c]

[a] CIBIO, Research Centre in Biodiversity and Genetic Resources, InBIO, Universidade do Porto, Campus de Vairão, Rua Padre Armando Quintas nº7, Vairão 4485 - 661, Vila do Conde, Portugal

[b] Departamento de Biologia, Faculdade de Ciências da Universidade do Porto, R. Campo Alegre, s/n, 4169 - 007, Porto, Portugal

[c] BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, 4485-661 Vairão, Portugal

* Corresponding Author: frederico.m.barroso@gmail.com

Index:

Introduction:

The novel methodology developed by Mochales-Riaño & Barroso et al. for experimentally obtaining thermal exchange rates of small lizards through the use of radiometric videos, underpinned the need to devise a thermal video processing protocol to standardize this data collection. This protocol addresses this need as it represents a subsequently optimized version of the processing pipeline used for the aforementioned proof-of-concept study.

The thermal videos mentioned hereafter were obtained with a FLIR thermographic camera (model T335, FOL 18 mm lens, sensitivity: < 0.05 °C; accuracy: ± 2%; IR image resolution: 320×240 pixels; FLIR Systems Inc., Wilsonville, Oregon, USA) and subsequently analysed using the *FLIR Tools* (version 5.13.18031.2002) software. Other brands and software do exist for capturing and processing thermal video. The proposed pipeline should be adjustable to these.

This protocol was used to successfully obtain temperature data from several body parts from thermoregulating lizards, enabling internal body temperature to be inferred (as per Barroso et al., 2016 and 2020), as well as providing insights into thermal exchange rates and the patterns of regional heterothermy and temperature redistribution in these animals.

So far, variants of this video analysis protocol have been successfully deployed in several ongoing research projects, working on the following lizard species:

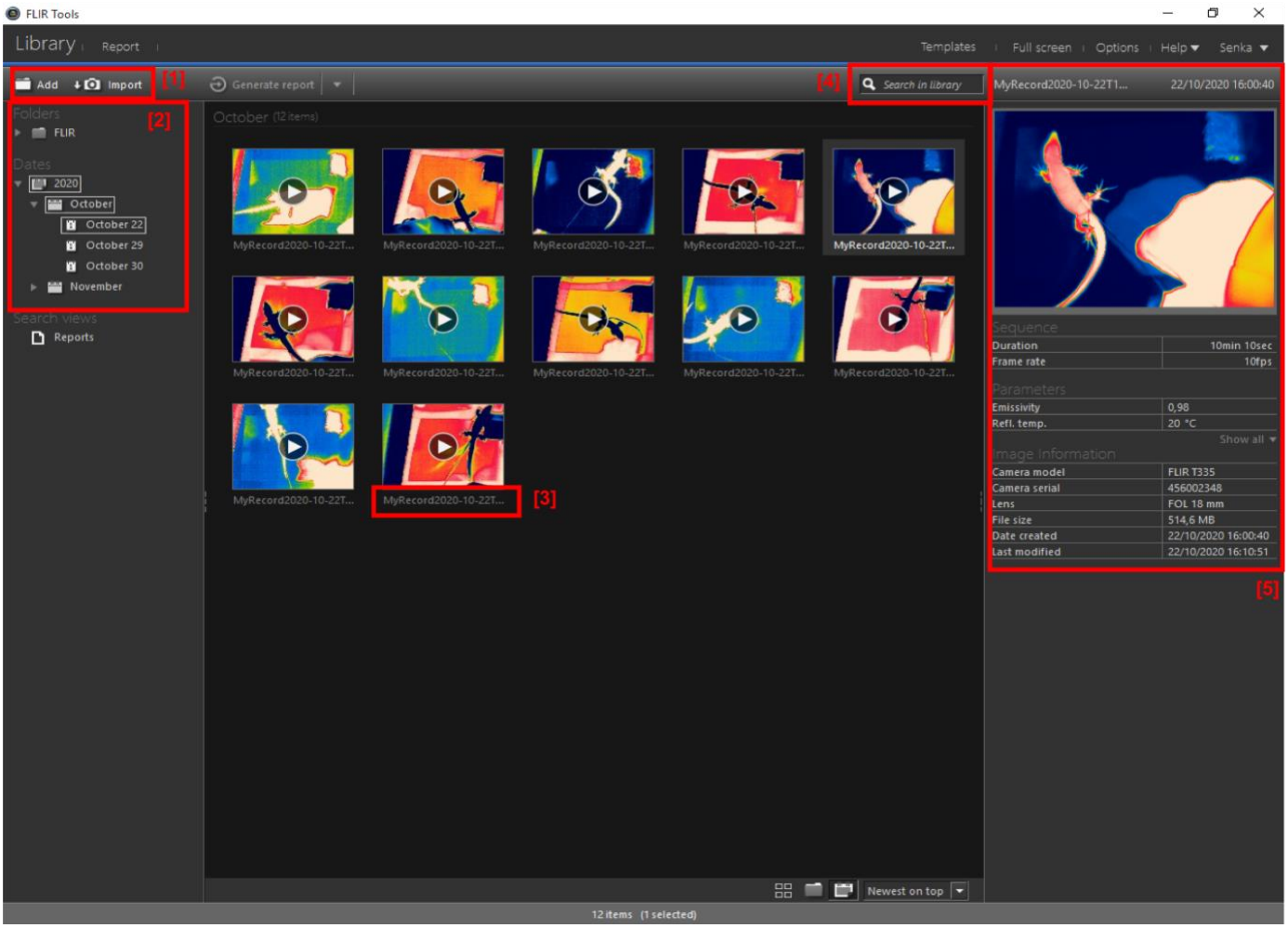
Species	*Snout-Vent Length (mm)	*Mass (g)	+Nr of Indiv.	+Year(s) of Data Collection
Tarentola mauritanica	70	9	20	2019/20
Podarcis bocagei	57	4	15; 21	2020/21; 2021
Podarcis lusitanicus	60	5	12; 85	2020/21; 2023/24
Podarcis virescens	58	4	10	2020/21
Podarcis carbonelli	51	3	14	2020/21
Podarcis siculus	76	10	9	2020/21
Iberolacerta monticola	72	9	12	2020/21
Teira dugesii	67	7	22	2021/22

* Overall mean mass and snout-to-vent length of the tested animals

+ Number of individual and year(s) for each project in which each species was tested

Getting Acquainted with the *FLIR Tools** Interface:

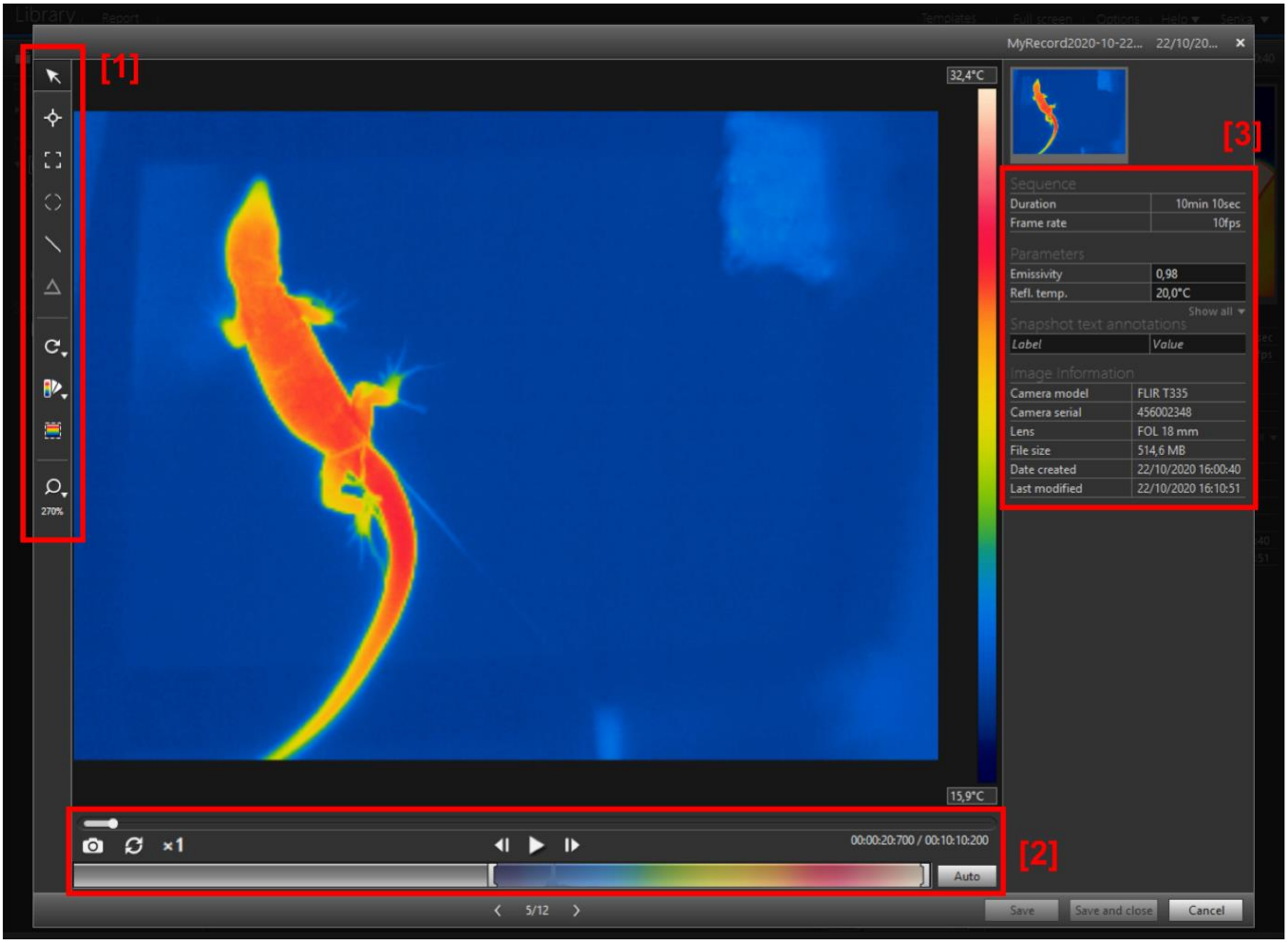
** The following guide (and presented figures/screenshots) applies to FLIR Tools and FLIR Tools+ **versions 5.x** for Windows operating systems. Other versions (older, Mac OS, mobile/Android) of this software may have similar interface and/or functions but exact layout may vary.*



This is the general library interface of FLIR Tools. It is where all the videos recorded or imported [1] can be found. These may be organized into folders [2].

The name of each file [3] is under its thumbnail preview. If the name is too long to be entirely visible, hovering the mouse over the thumbnail the full name will appear. Alternatively, there is a search option [4] to look for specific files.

The right column [5] shows a preview of the selected file as well as the associated metadata (e.g. cameral model, date of capture, etc.)



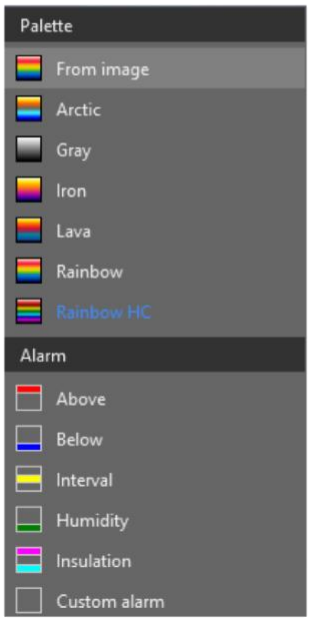
Double clicking on a video file will prompt the video edit window, overlaying the library window.

On the left panel [1] there are the editing tools (i.e. obtain measurements from the video, choose the false-colour palette, etc).

On the bottom panel [2] are the video control tools such as the progress bar (which can be shuffled to manually move to a specific location along the recording), the play/pause button as well as the adjustable temperature scale colour bar (which can be left in the pre-set "Auto" adjustment or move the range all together or the minimum or maximum bracket for finer control of temperature visual resolution). It is important to note that changing the scale colour does not alter the recorded temperatures, only the visualization (i.e. the range of the mapped false-colour scale) is changed.

On the right panel [3] are the image information, the parameters (which are editable and will affect the temperature readings) as well as eventually the measurements, once the *Tools* are used to obtain these (not visible in this screenshot since no measurement had been taken yet).

THE TOOLS:



Add Spot Measurement: tool to measure the temperature at a given pixel of choice

Add Box Measurement Tool: create a box of any desired dimensions from which the temperature of every pixel can be extracted or summarized in *Measurements Panel* into min, max and average (mean) temperature

Add Ellipse Measurement Tool: Similar to box measurement tool, but creates a circle/ ellipse instead

Add Line Measurement Tool: create a line, of 1px by any desired number of pixels, from which the temperature of every pixel can be extracted or summarized in *Measurements Panel* into min, max and average temperature.

Add Delta Function: Function to compare temperature values between measurement tools

Rotate Image and Measurements: Rotate the image

Select Among Colour Palettes: Select from a range of colour palettes

Set Auto Adjust Region: Allows the user to define a rectangle around the area to which the temperature/colour range should adjust to maximise temperature visual resolution. Often useful to define the region around the lizard, avoiding hot/cold spots elsewhere.

Zoom: Select the amount of magnification on the image. Use CTRL +/- to zoom in/out, use SPACE key and mouse to pan or use ALT key and drag mouse to select an area to zoom in to

THE VIDEO CONTROLS:

Video Progress Bar: Tracks the elapsed time in the video. The sphere can be shuffled back and forth to find a particular location/frame along the recording

Total Recorded Time

Elapsed Time

Play/Pause

Back: Go back one frame

Forward: Go forward one frame

Adjust Playback Speed

Repeat

Save a Single Snapshot as .JPEG: Save the frame as a still image

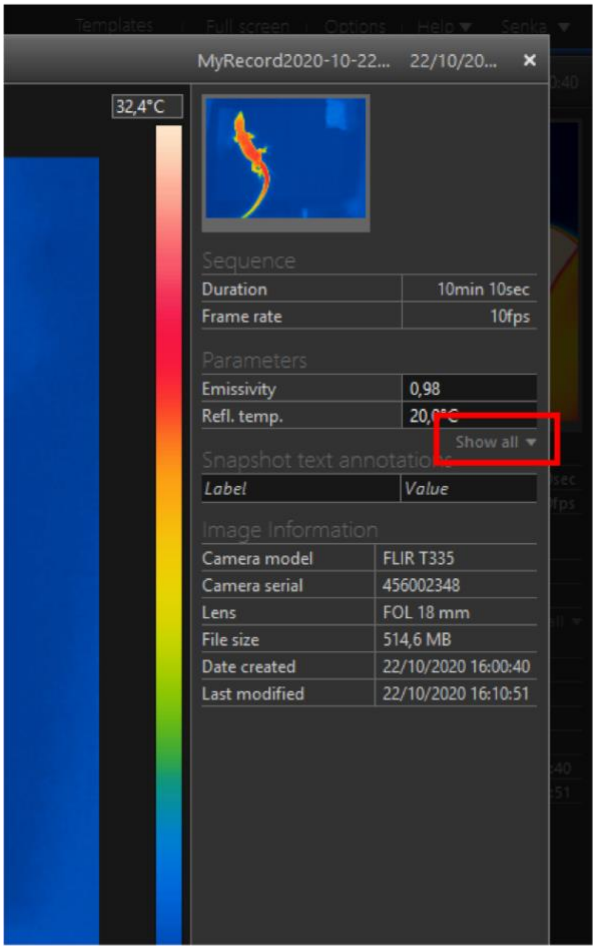
Select and Slide for Minimum Adjustment: Adjust the minimum temperature of the temperature/colour interval range

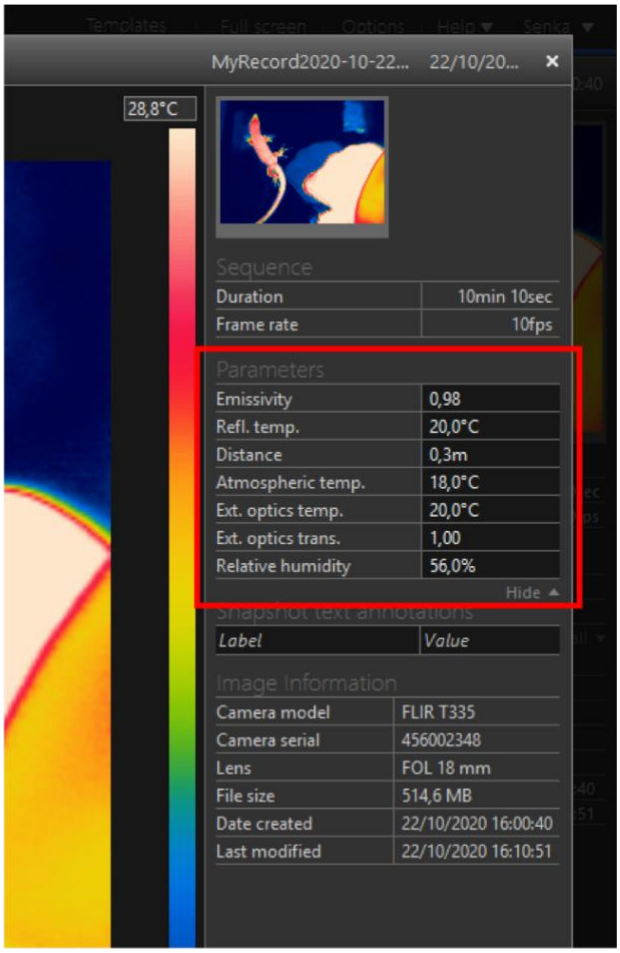
Click and Slide to Move Interval: Adjust a fixed range of temperature/ colour interval

Select and Slide for Maximum Adjustment: Adjust the maximum temperature of the temperature/colour interval range

Automatic Adjustment of Interval: Automatically adjust the temperature/colour interval

The *Parameters* for the frame must then be checked. *Usually*, most of the parameters are hidden. Hence the first step is to expose these in "**Show All**". These are all editable and will either have preset values or, in some cases, be measured by the camera during recording (also depends on the camera model).





***Note**: these images are from different frames (i.e. different times) of the same video, hence the differences in what is seen in frame*

The most relevant parameters, which must be checked at the start and/or throughout the video analysis, are:

- *Emissivity:*
  - *Emissivity ($\mathcal{E}$)* is the ratio of infrared energy emitted by the object compared to that emitted by an ideal blackbody, if both are at the same temperature. The ratio is on a [theoretical] scale from 0 (perfect infrared mirror, reflects all IR radiation) to 1 (perfect black body, absorbs/emits all IR radiation).
  - Skin, in general, is known to have an emissivity in the 0.95-0.98 range, but previous thermography studies on lizards have a adopted an emissivity of 0.96 (e.g.: Barroso et al., 2016, 2020). Hence, for consistency and inter-study comparability, $\mathcal{E}$ = 0.96 is recommended for further thermography studies on lizards and adopted for the remainder of the protocol.

- [Apparent] *Reflective Temperature:*
    - Thermal radiation, from other external sources, reflecting on the subject/object being measured. This must be corrected for in order to obtain accurate temperature readings.

- *Distance* (from the camera to the subject, in meters).
    - Distance between the camera and the subject must be measured to account for the attenuation of infrared radiation by the environment (Faye et al., 2016). Realistically, in practice, this should only matter at the magnitude of a distance of several meters but should nonetheless be considered.

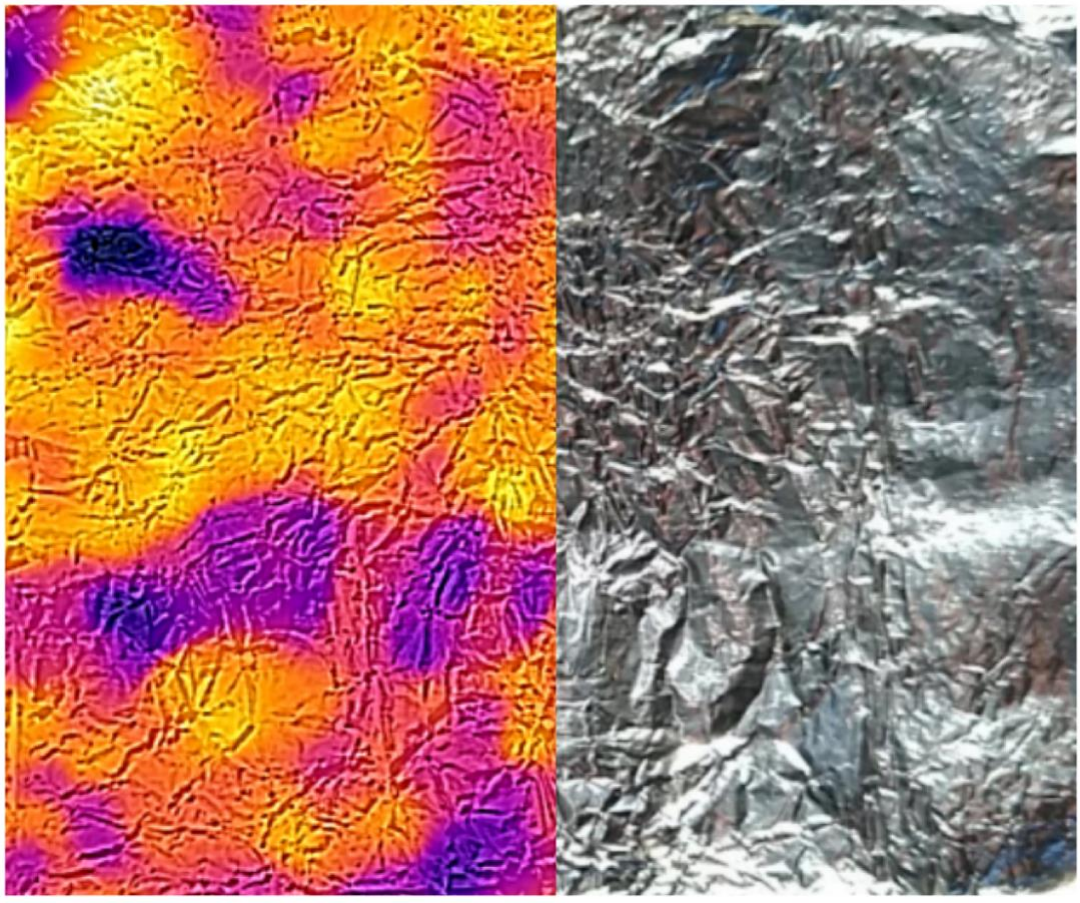
Some secondary parameters must also be considered and checked as well. These are usually fine left at the pre-set/ camera-measured values (unless deviating a lot from the reality) but it is important to pay attention to their consistency throughout the video analysis. These are: *Atmospheric temperature* (usually 18ºC pre-set and maintained for this protocol) and *Relative humidity* (pre-set 56% and maintained for this protocol).

All other parameters must be kept constant and are **usually** fine left to the pre-set/ camera-measured values.

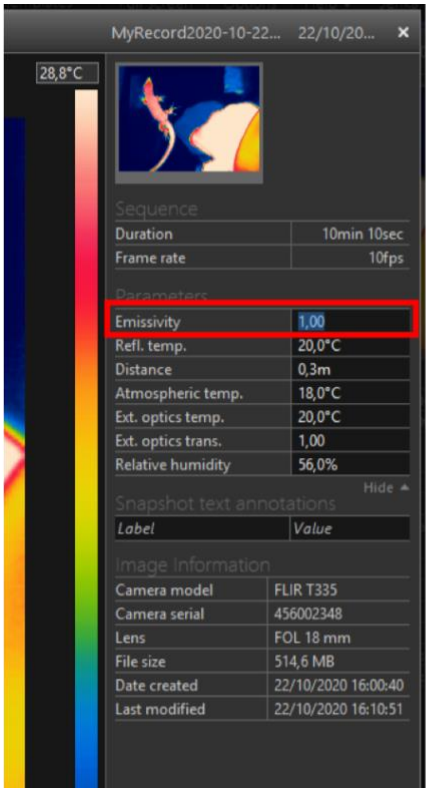
Measuring and Correcting for the [Apparent] Reflective Temperature:

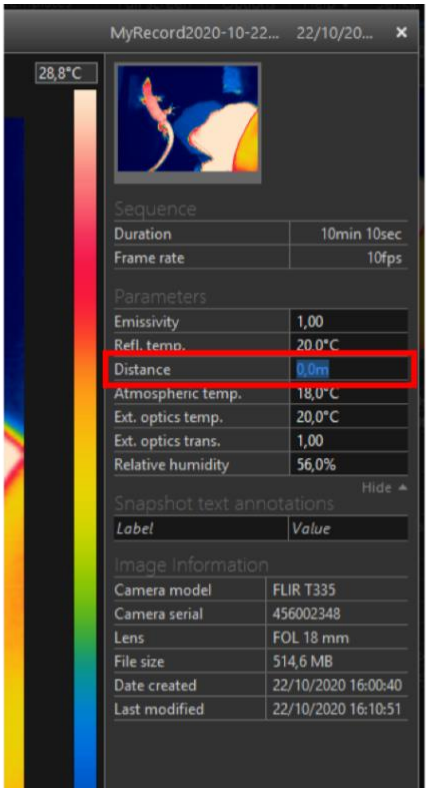
Reflective (or reflected/ or background) temperature relates to the background thermal radiation, from surrounding heat sources, that gets reflected from the subject's surface. Since this is not infrared radiation being emitted by the animal/subject itself (it is radiation being reflected by it instead), it must be discounted from the final thermal reading in order to obtain an accurate measurement of the actual temperature of the subject. This can vary considerably along time, as surrounding conditions around the subject change constantly. Hence, it is important to constantly measure and correct for it throughout the analysis of each video and of each frame.

To measure this parameter, a rectangle of wrinkled aluminium foil (IR vs RGB image below) was maintained in the frame of the recorded video. Given its polished metal surface it will be a good approximation to an infrared mirror, and the wrinkles on its surface should reflect IR radiation in the environment incident from several angles. Ultimately, it should provide a good approximation of the available sources of IR radiation that may also be reflecting from the subject of interest. Hence, by assuming that the measured average temperature of this aluminium foil comes entirely from the reflective IR sources, one can obtain an acceptable approximation of this parameter which can then be used to correct the temperature measured in the subject of interest.



To measure the *Reflective Temperature* using the aluminium foil method, in the [*Global*] *Parameters* panel, change the *Emissivity* value to **1.00** and the *Distance* value to **0.00m**. In addition, confirm that the *Atmospheric temp.* and *Relative humidity* are the established [constant] values (18ºC and 56% respectively).





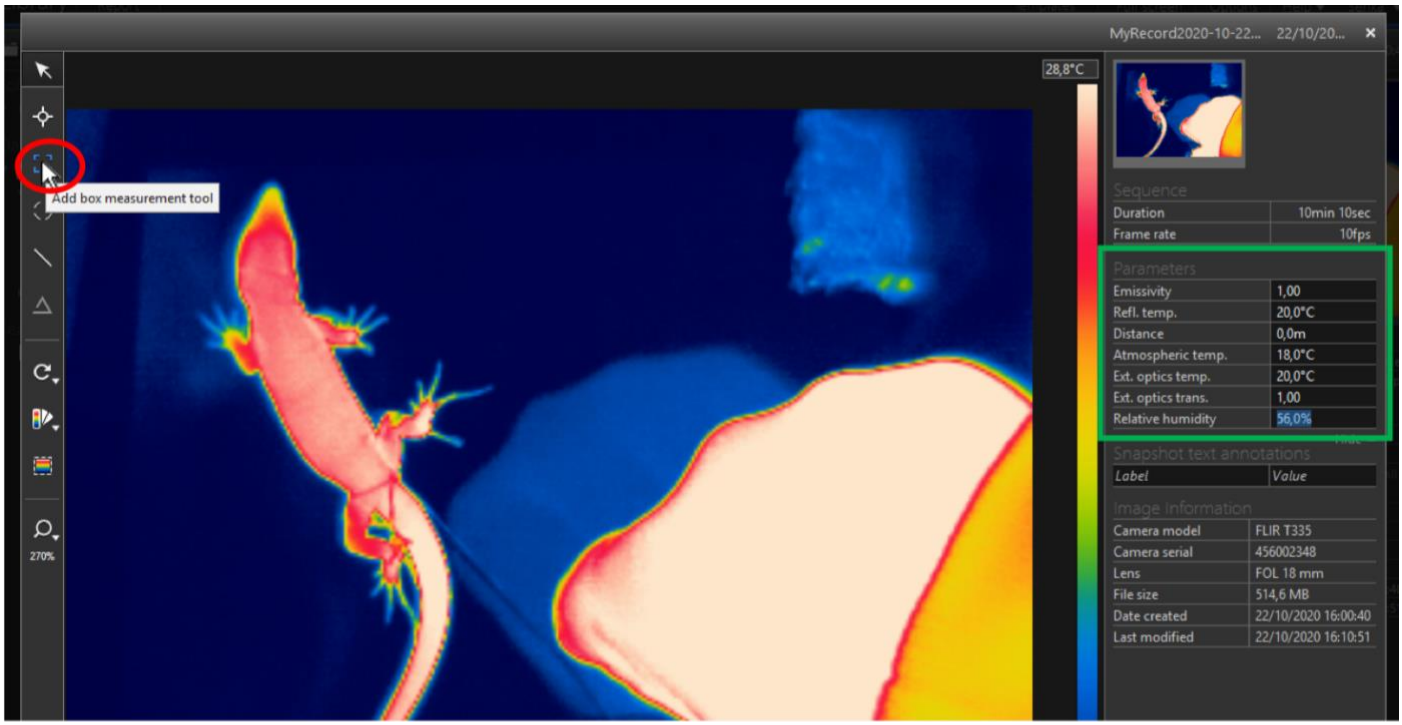
CHANGE:

- Emissivity to **1.00**
- Distance to **0.0 m**

CONFIRM:

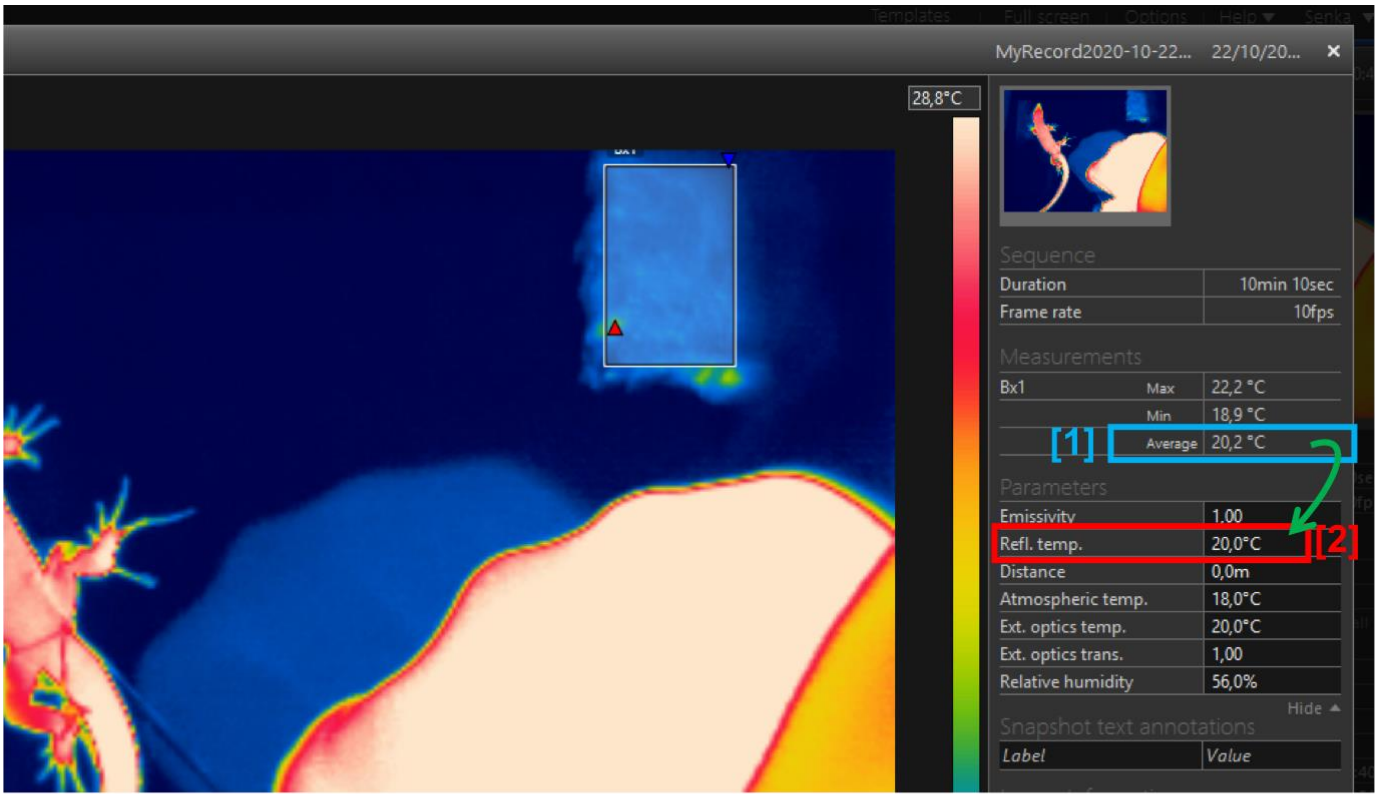
- Atmospheric temp = the defined (constant) value (18 ºC)
- Relative humidity = the defined (constant) value (56.0%)

WARNING: Having these set in the *Global Parameters* means that any measurement applied will also be subject to these values, which may not be the intention (but see chapter "Setting [Fixed] *Local Parameters*").



KEEPING THE PARAMETERS:

- Emissivity = **1.00**
- Atmospheric temp = the defined (constant) value (18 ºC)
- Distance = **0.0 m**
- Relative humidity = the defined (constant) value (56.0%)



Create a box encompassing as much area of crinkled aluminium paper as possible but **without** including any of the surrounding area/material (or any visibly anomalous hot or cold spots)

The measured *Refl. temp* = *Average* temperature of *Bx1*

Add a *box measurement tool.* Create this box encompassing as much area of crinkled aluminium paper as possible but without including any of the surrounding area/material (or any visibly anomalous hot or cold spots).

The average measured reflective [apparent] temperature will be the measured average temperature of box *Bx1* [1]. The *Refl. temp*. field [2] in the *Parameters* can now be updated with the measured value.

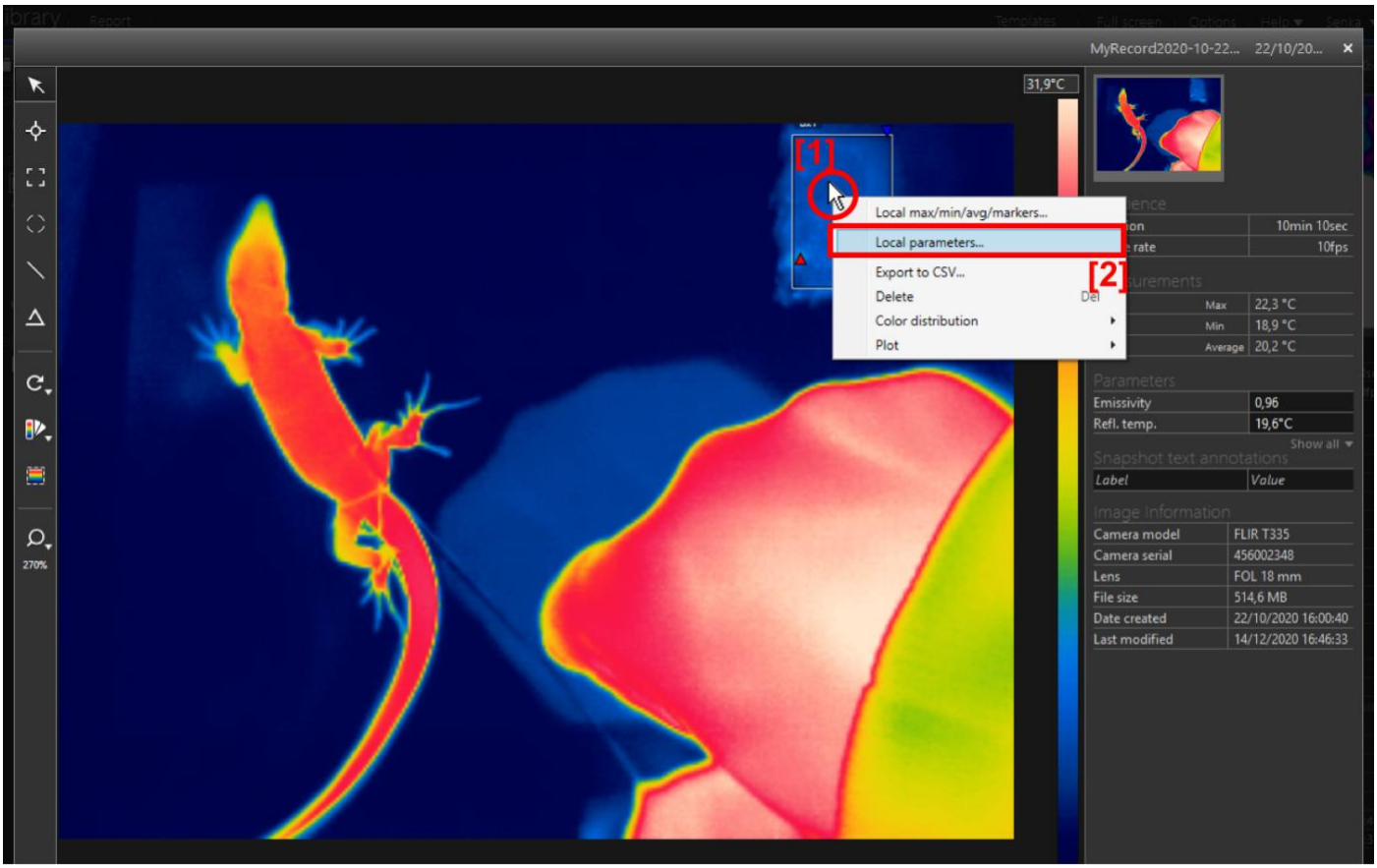
However, it is important to note that this only applies to the specific conditions found in that particular frame/photo. Although much variation is not expected given the small, 20 second, interval between data points and the fact that, for these experiments, external conditions where maintained as stable as possible, the reflective temperature should nonetheless be confirmed/measured at each sampling frame. This can more efficiently be done through fixing the ***local parameters*** ($\mathcal{E}$ = 1 and distance = 0) of the box. Setting local parameters allows the box to have a different set of parameters to those of other measurements (i.e. emissivity, distance, etc. parameters can be reset to other values, specific for the subject without affecting the measurements in *Bx1*).

Setting [Fixed] Local Parameters:

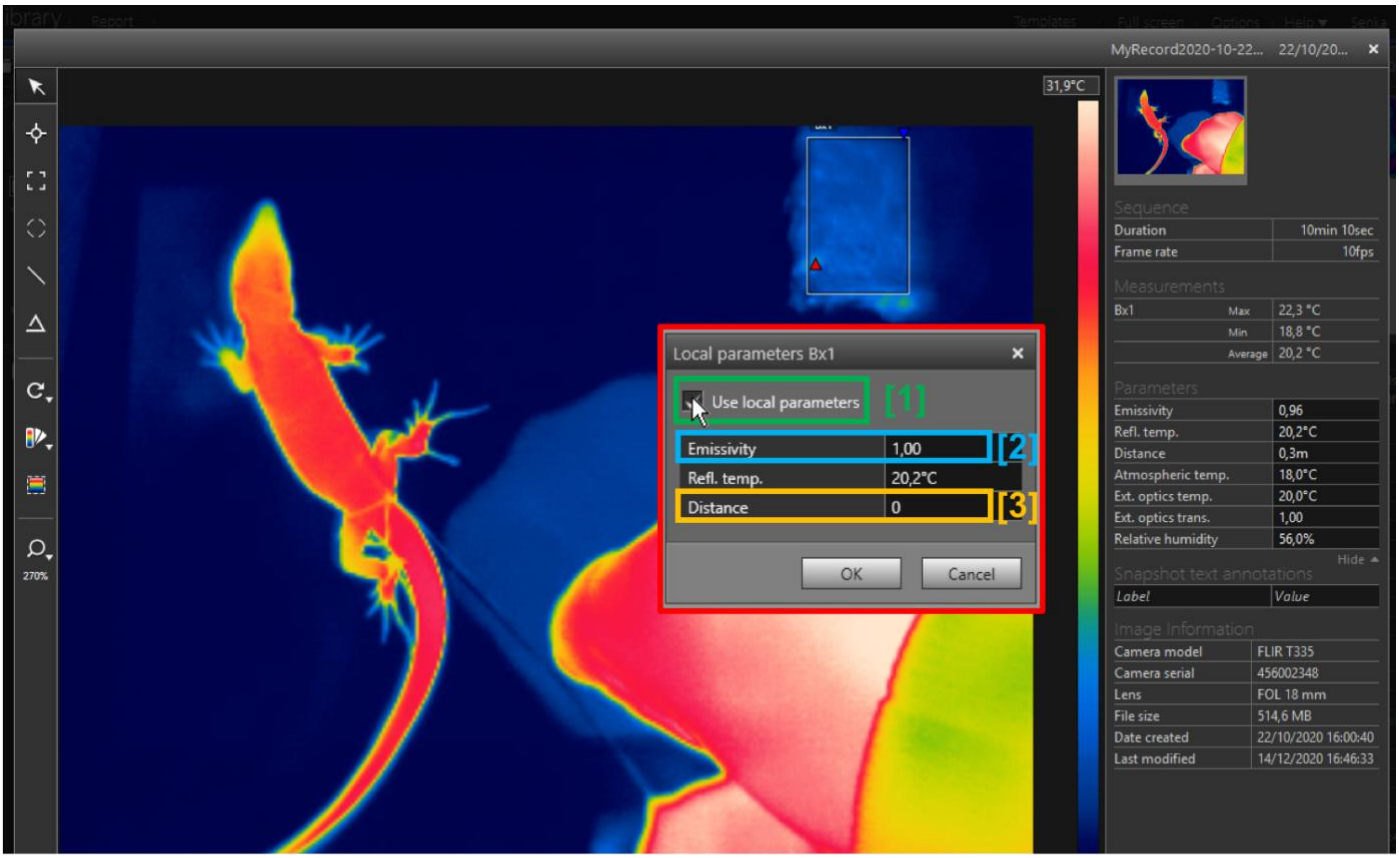
This allows setting specific parameters (emissivity, distance and reflective temperature values) for any specific measurement (box, spot, etc…). Different *local parameters* can be set on each measurement or for only a specific measurement, while global parameters (on the right pane) will be adopted for the rest of the measurements to which *local parameters* have not been attributed.

FLIR Tools will always use the *global parameters* for any new measurement added unless one overrides it by specifying *local parameters* for that particular measurement. Additionally, changing the *global parameters* will have no effect on the *local parameters* of any measurement where these have been specified.

For a video, whatever *local parameter* for a particular measurement is set in a frame, will be carried forward (or backward) for that that particular measurement in all other frames.



To set a *local parameter*, add a measurement (any will do: *box, spot*, etc.) and right click on anywhere inside the measurement where these are to be set[1] (in this case, *Bx1*). Then select *"Local parameters…"* from the drop-down menu [2]. This will prompt a pop-up window.

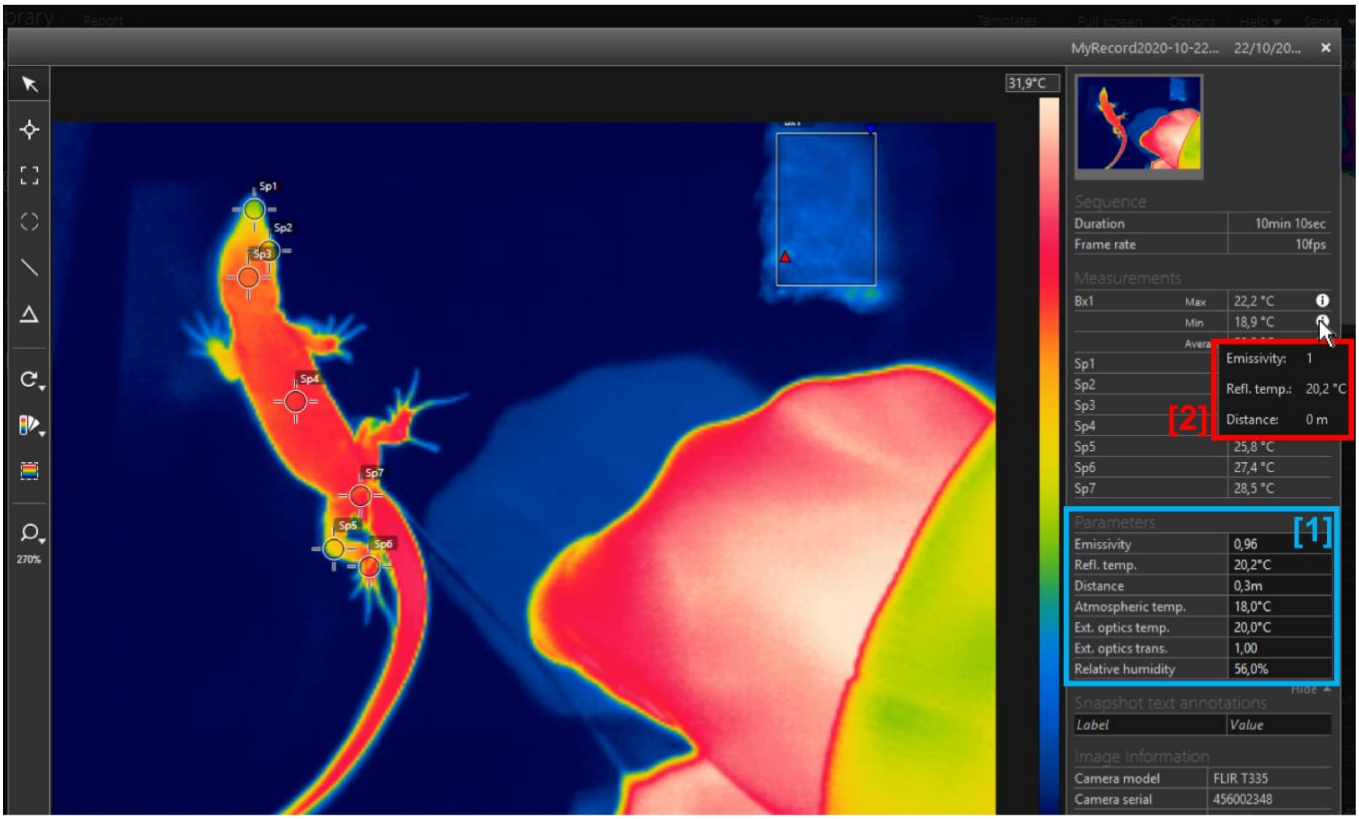


SET THE Bx1 LOCAL PARAMETERS:

- Emissivity = **1.00**
- Distance = **0.0 m**

(Ignore the *Refl. temp.*)

In the pop-up window, select ("Tick") the *"Use local parameters"* box [1] and set the ***Emissivity*** to **1.00** [2] and the ***Distance*** to **0.0m** [3]. The ***Refl. temp.*** can be ignored in this case (when $\mathcal{E}$ = 1 and **D** = 0) and so it can be left at whatever value it was established in the [*Global] Parameters* (on the main, right pane).

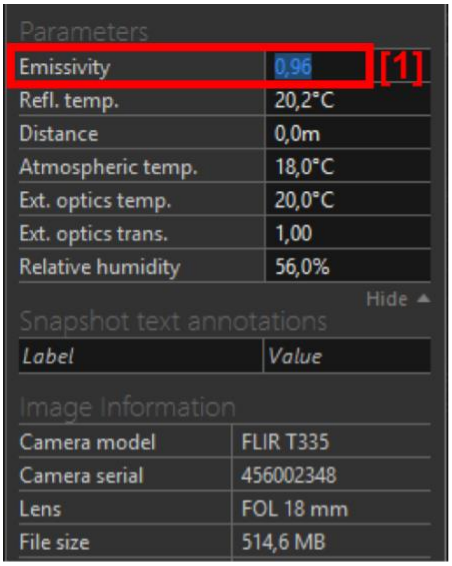


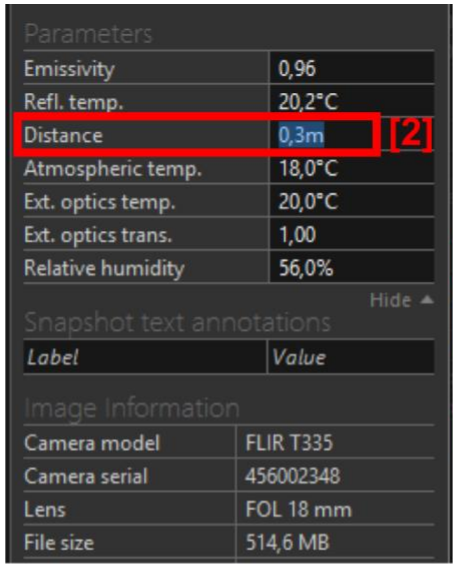
Now adding any measurements will adopt whichever parameters are set in the [*Global*] *Parameters* pane [1]. These may or may not be different to the *Local parameters* set for *Bx1.* Changing the *[Global] Parameters* will not affect the *Local Parameters* set for *Bx1* (and vice versa). Different measurements may have different *Local Parameters,* and whichever measurements are not specified local parameters, will always follow the *Global Parameters* set.

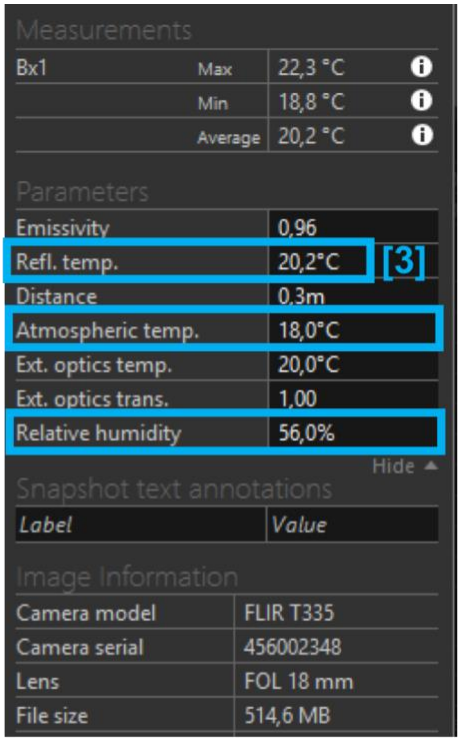
Notice that an "*i*" appears next to the measurements of *Bx1.* This is to warn the user that the parameters of this measurement are different from those of the *[Global] Parameters.* Hovering the mouse over the "*i*" will show what are the specific *Local Parameters* [2] set for that measurement.

Setting the Correct [Global] *Parameters* for the Animal (and Substrate) Temperature Measurements:

It is important to note that different experimental settings may require different parameters. These may be adjusted in the *Global Parameters* menu. For the purpose of the described protocol, developed by Mochales-Riaño and Barroso et al. (in press) to study lizard thermal exchange rates, these are the parameters defined:







CHANGE:

- Emissivity to **0.96**
- Distance to **0.3 m**

CONFIRM:

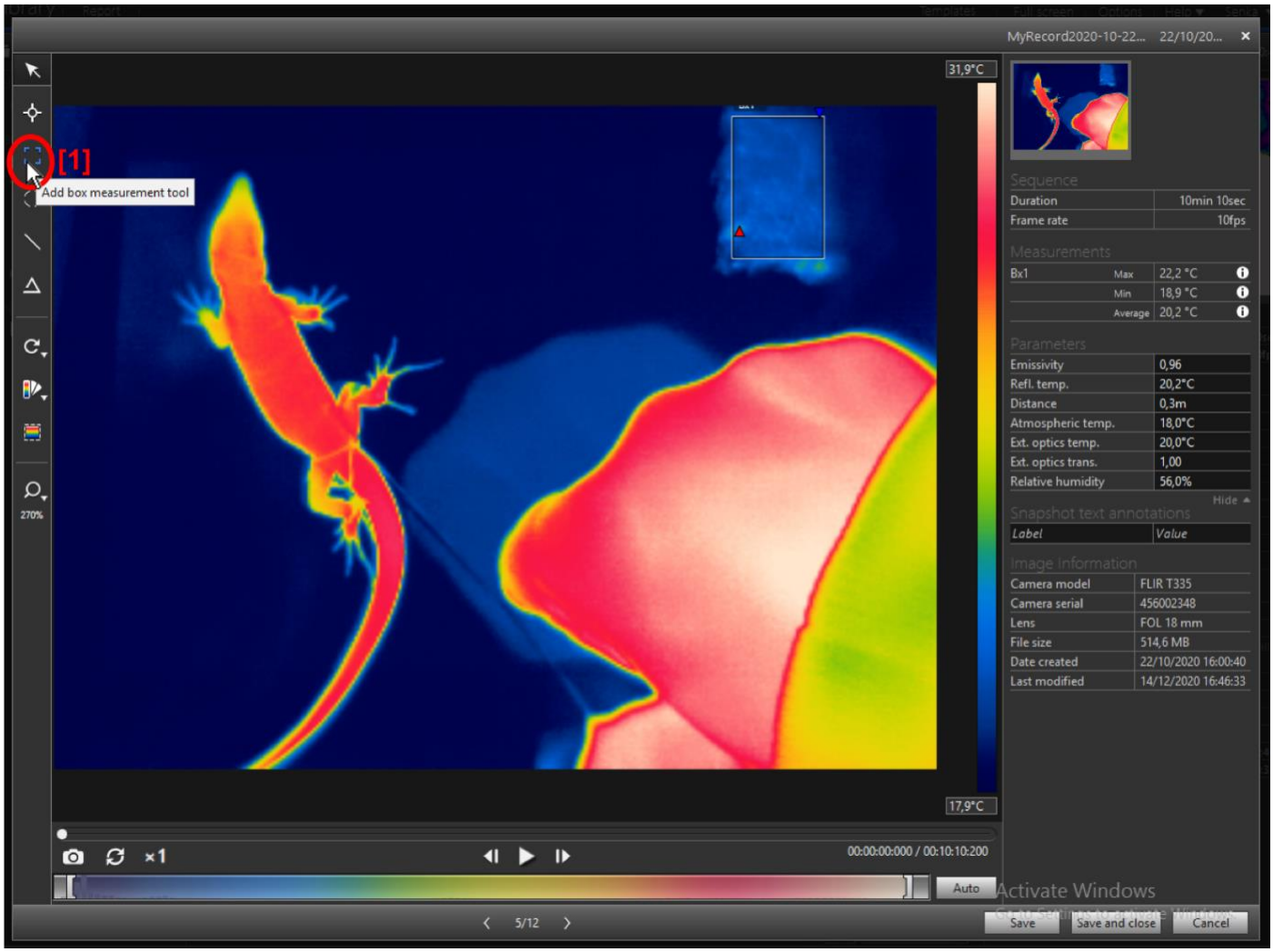
- Atmospheric temp = the defined (constant) value (18 °C)
- Relative humidity = the defined (constant) value (56.0%)
- **Refl. temp. = Bx1 Average [temp]**

In the [*Global*] *Parameters* pane, change the ***Emissivity*** to **0.96** [1] and the ***Distance*** to **0.3m** [2]. Additionally confirm (and change if necessary) the ***Refl. temp.*** value is the same as the ***Bx1 Average [temperature]*** <u>for that specific frame</u> [3] and that the *Atmospheric temp.* and *Relative humidity* values remain at the [constant] pre-determined values (18ºC and 56% respectively)

Measuring the Substrate [Surface] Temperature:

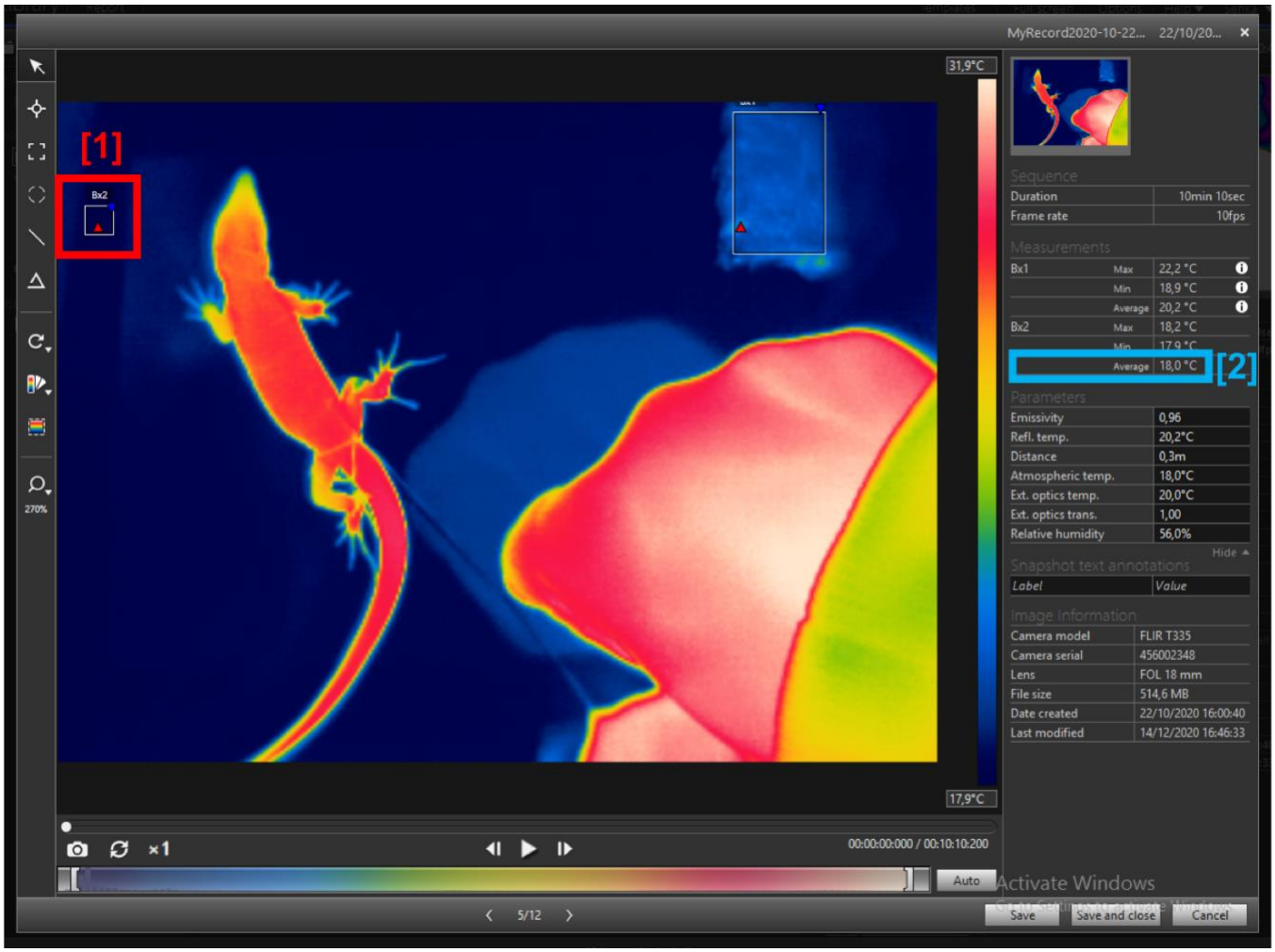
Black electrical tape of known emissivity ($\mathcal{E}$ = 0.96) was used to delimit the edges of the experimental arena. Since this tape was directly stuck to the substrate (the glass of the aquarium) it can be assumed to be isothermal (i.e. same temperature) as the substrate/surface itself. Hence, it can be used to infer the temperature of the substrate. This is more accurate that measuring the temperature from the glass surface since glass has lower emissivity and thus higher reflectivity than the black electrical tape, increasing potential error in the measurement. Additionally, the tape has the same emissivity as the skin (0.96) reducing the need to set *Local parameters* for this measurement.

For this, once again add a *box measurement tool* [1].



A measurement box [1] must be created in an area of tape that does not show any abnormalities (wrinkles, unusual hot/cold spots/ reflections) or which is not often obstructed by the animal. Corners of the arena should be avoided as this is where two strips of tape overlap. The box must be entirely over the tape so edges of the tape must also be avoided, to prevent the effect of some image distortions.

Once this is done for the first frame (time = 0 seconds), the box can be left in place for subsequent frames (assuming the set-up does not move). However, if the animal moves and obstructs partially/fully the box or if abnormal hot/cold spots appear, the box must be moved to another, more suitable location, as close as possible to the original location.

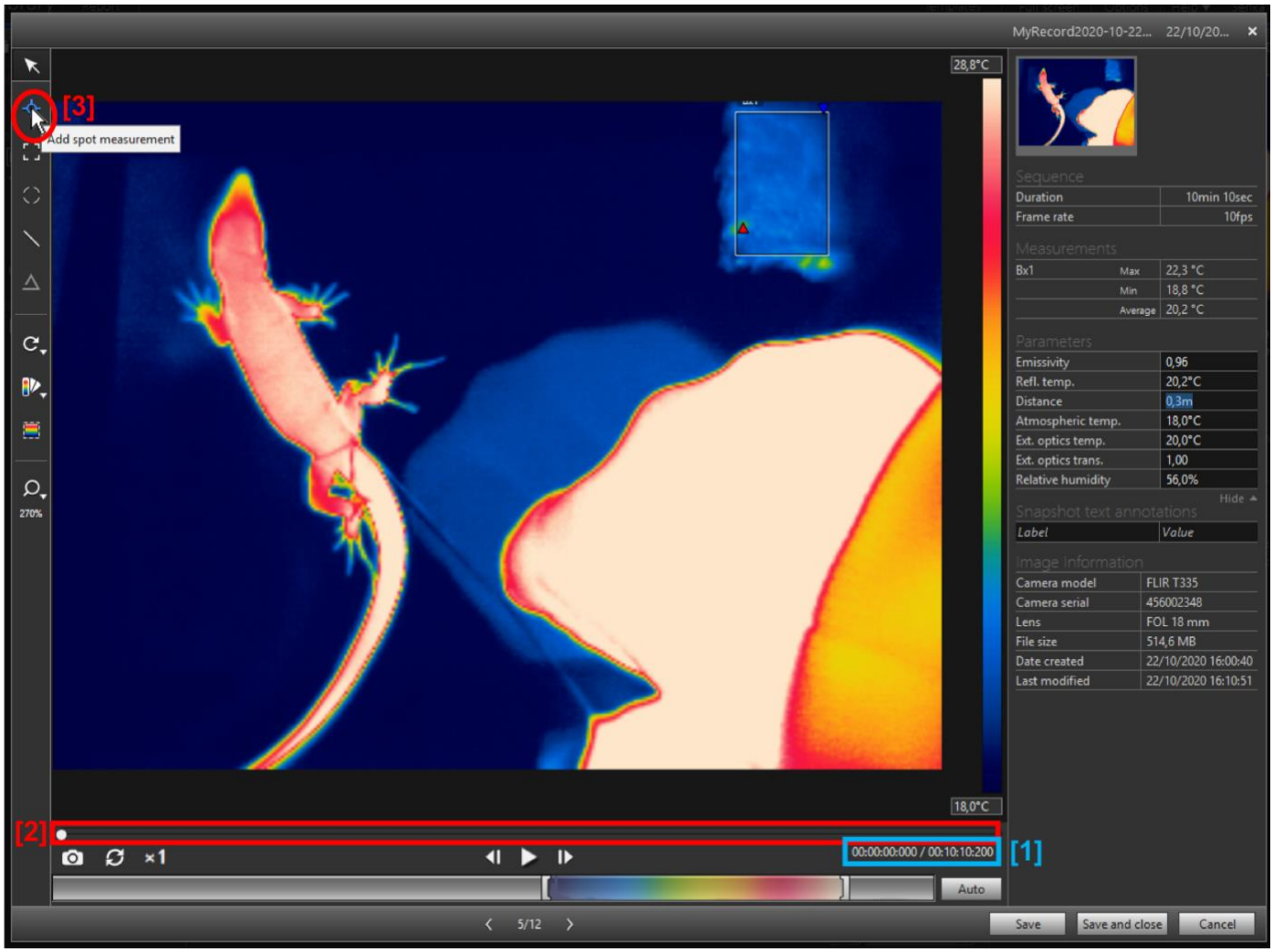


The temperature of the substrate will be the ***Average*** temperature of *Bx2* [2]. The measured value in each data collection frame (every 20 seconds) must then be recorded in an external datasheet/database (See "Example Database for Recording the Data").

[Note that Bx2 does not have the "i" sign, this is because it is assuming the [Global] Parameters as opposed to Local ones (which would need to be set manually as was done for Bx1)]

Measuring the Animal's Body Temperature (at Several Body Locations):

For the purpose of the novel protocol developed by Mochales-Riaño and Barroso et al., the temperature of several specific body parts of the animal were required. Future applications of the protocol are nonetheless flexible as to which body parts need to be measured according to the user's requirements.



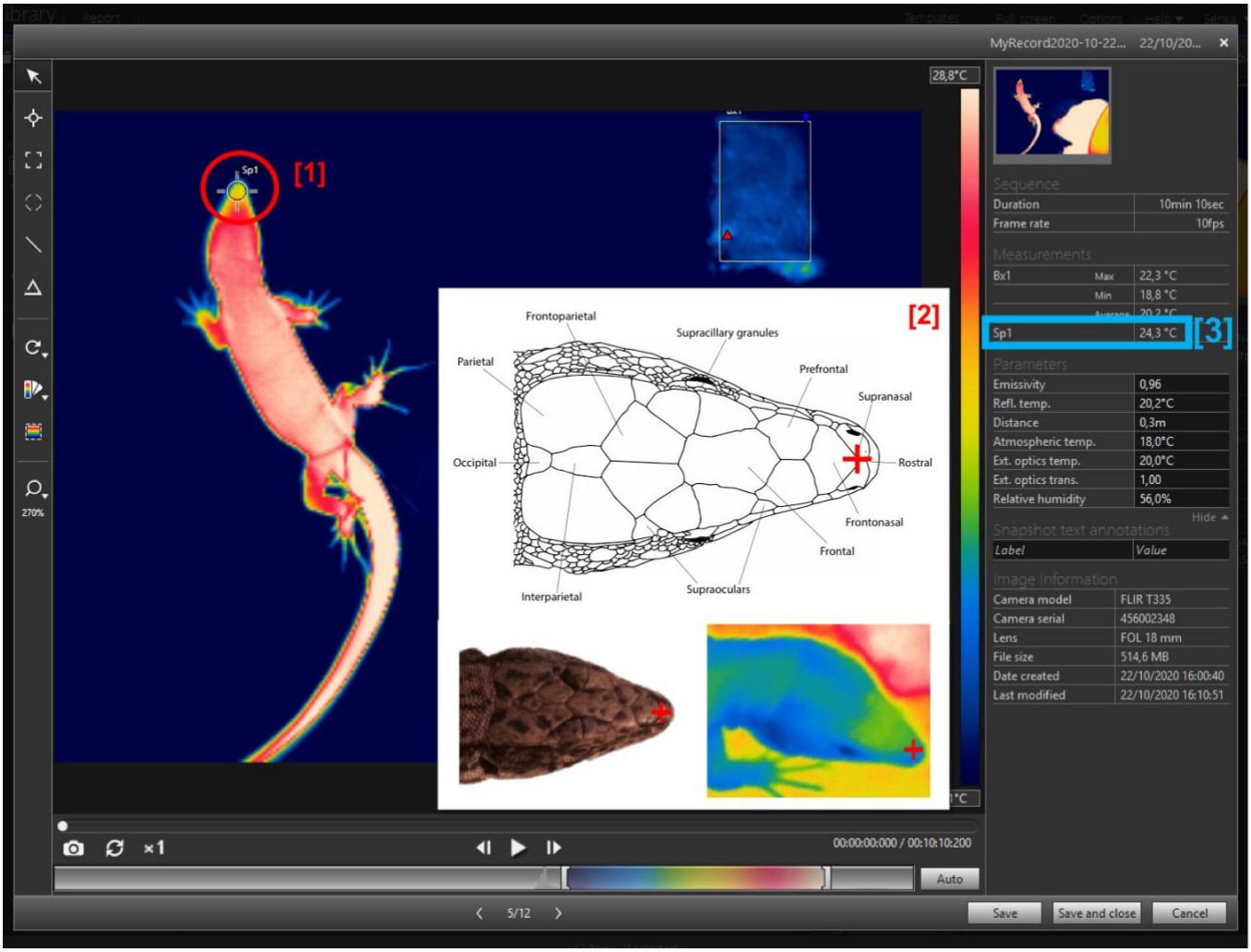
First it is important to confirm that the video is in the desired frame for obtaining measurements. This is done by looking at the video's elapsed time [1]. For the studies by Barroso et al., measurements were obtained every 20 seconds, starting at time = 0 seconds (the frame in the screenshot). If not at the desired time, either "play" the video until the desired time is reached or use the video progress bar [2] along with the elapsed time clock to find the appropriate frame.

Once at the appropriate frame, the *Spotmeter* tool [3] will be used. This will allow the placement of a measurement (with the size of 1x1 pixel) at any location of the photo. For increased precision, once the *Spotmeter* tool is selected, use the mouse to find the desired location **before** placing the measuring pin. This will give a finer control than just "dropping the pin" anywhere and then trying to move it to the best location. If a mistake is made, or the spot chosen is deemed unfitting, it is always best to delete the pin (click on the pin + "Delete" key) and place a new one than to trying to move a misplaced pin around.

For the purpose of the associated studies, body temperatures were measured at 8 locations in the following order: **snout**, **right eye**, **left eye**, **head**, mid-**dorsum**, **left leg** (knee), **left palm**, **tail** (dorsally, above the location of the cloaca**). If any such location(s) is not visible in a particular frame, the measurement for that body position at that frame is simply skipped**.

NOTE: The use of the *Spotmeter* tool is controversial and has had some criticism from reviewers in past studies. This is because this tool only takes into account 1 single pixel, thus making it more susceptible to catching noise in the data (i.e. that pixel may not be representative of the pixels around it and may therefore not be a good representation of the average temperature of the measured body part). Hence, it is arguably better to use the *Box* or *Ellipse measurement tools* since these take into account the average temperature over an area. However, give the small size of some of the areas of interest (e.g. snout, eye, palm) drawing a box or ellipse that encompassed **only** the desired body part would prove very difficult. Furthermore, we would need to make sure that the area of such box or ellipse would be kept constant throughout the analysis, adding another layer of complexity to an already laborious task. Hence, for simplicity and efficiency, we decided to continue using the *Spotmeter* tool as previous studies provided adequate and representative results with this tool (Barroso et al 2016, 2020).

*[**DISCLAIMER:** Please ignore the fact that Bx2 (substrate temperature) and the "i"s in Bx1 measurements are missing (i.e Bx1 is using Global as opposed to Local Parameters, and the substrate temperature was not measured). These images are purely for illustrative purposes specifically regarding placement of the Body Temperature points.]*

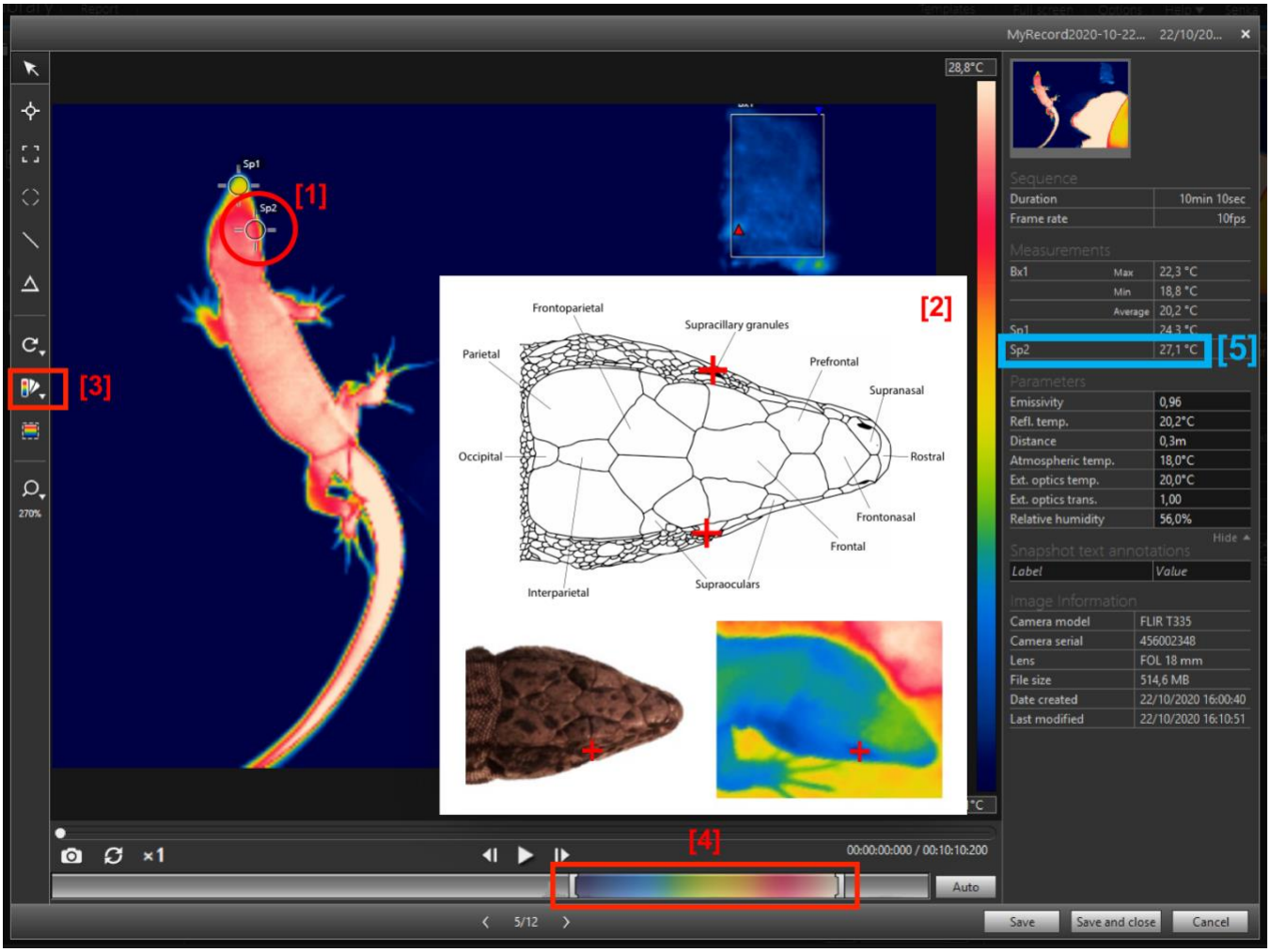


Spot 1 [1] will be placed at the snout [1,2]. Before "placing the pin" use the cursor (shaped like a cross) to find the approximate location of where the mid-point of the two nostrils would be (approximately in the intersection between the frontonasal and supranasal scales – see diagram). Remember that the nostrils are not at the very tip of the snout. Note the position of the nostrils and where to place the pin (red cross) in the example photos and diagram [2].

Additionally, avoid placing any pin close to the edge of any body part/ structure, as edges are often prone to distortions and other image aberrations leading to incorrect temperature readings.

Also consider the fact that the head might be tilted. This may require you to place the pin slightly of-centre to compensate for the tilt.

Record the value for ***Sp1*** [3] in the appropriate datasheet/database's column <u>before moving to the next frame</u>.



Spot 2 [1] will be in the **RIGHT** eye. Use the cross-hairs of the cursor to find the centre of the left eye and place the pin as centrally as possible. As with other measurements, if unhappy with the placement, delete the spot (select the spot + press *Delete*) and try again. **Do <u>not</u> try to adjust the location of the dropped pin**.

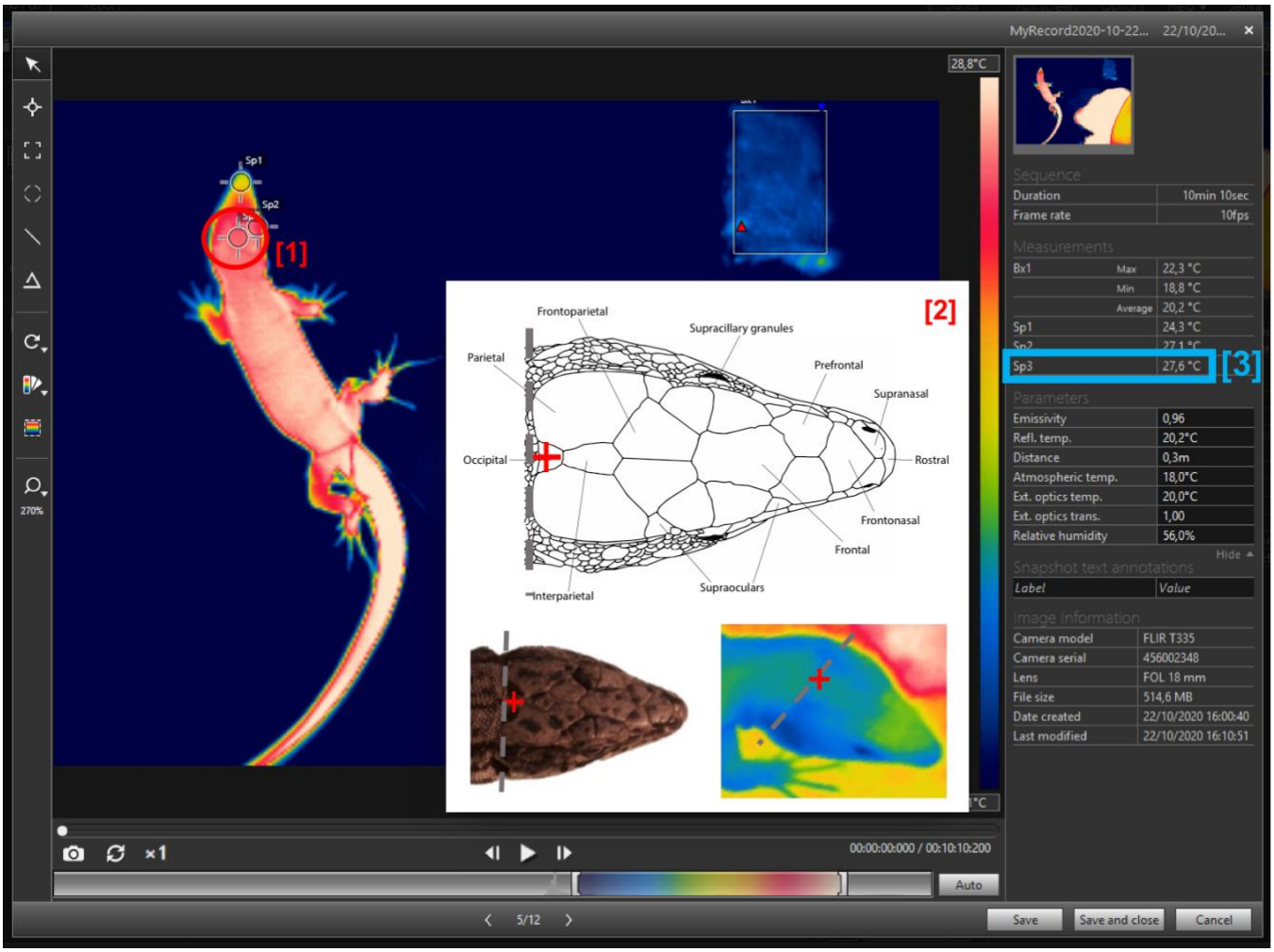
Spot 3 **WOULD** be placed in the **LEFT** eye (which is not visible in this frame and hence the measure is skipped <u>for this frame</u>) following the same process as for Spot 2.

Again, bear in mind the possible tilt of the head when looking for the position of eyes. Check the position of the eyes, and where to drop the pin (red cross) in the diagram [2].

Often one or even both eyes might be difficult to find. To better locate the eyes there are a few tricks/ combinations of tricks one can use:

- Change the false-colour pallet - *Rainbow HC* and *Arctic* provide good range of contrasting tones/colours and so are often useful [3];
- Adjust the temperature/colour interval [4] – often the eye becomes obvious during the process of adjusting (while you are moving the controls), not necessary at any one single setting;
- Rely on the knowledge of where the eye should be in terms of the lizard's head morphology (e.g. in relation to the snout or the end of the head, in line with the other eye which may be more visible, etc.). refer to the position of the eye in the example photo [2].

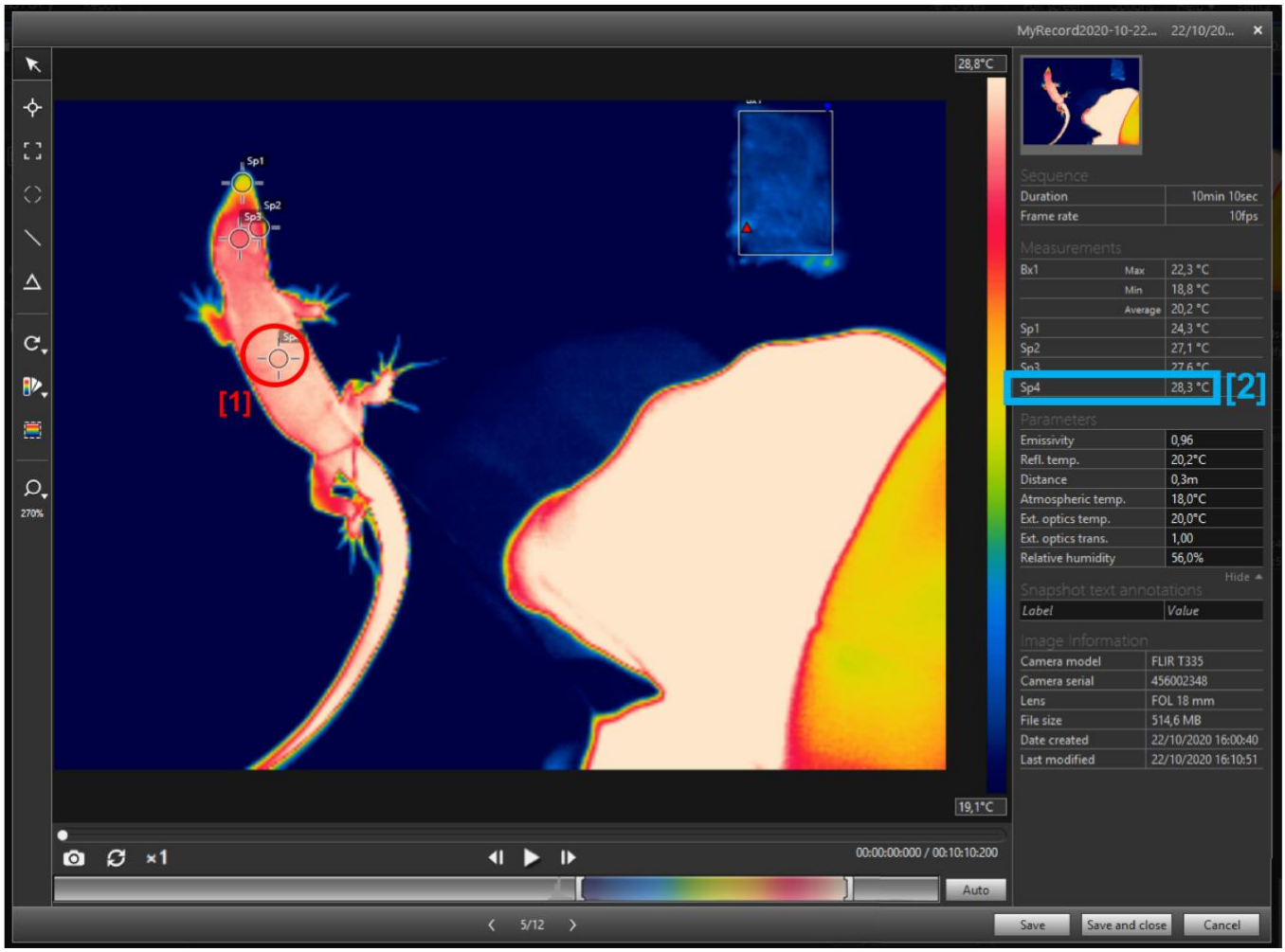
Record the values for ***Sp2*** [5] (and the eventual ***Sp3*)** in the appropriate datasheet/database's column(s) <u>before moving to the next frame</u>.



The 4th measurement will be of the **head**. To do so a spot must be placed **at the base of the head above the location of the occipital scale** [1,2]. Often, the easiest way to locate this is to identify the base of the parietal scales (dotted, grey line in the diagram [2]) and placing the pin centrally along that line.

As with other measurements, if unhappy with the placement, delete the spot (select the spot + press *Delete*) and try again, **do not try to adjust the location of the dropped pin**.

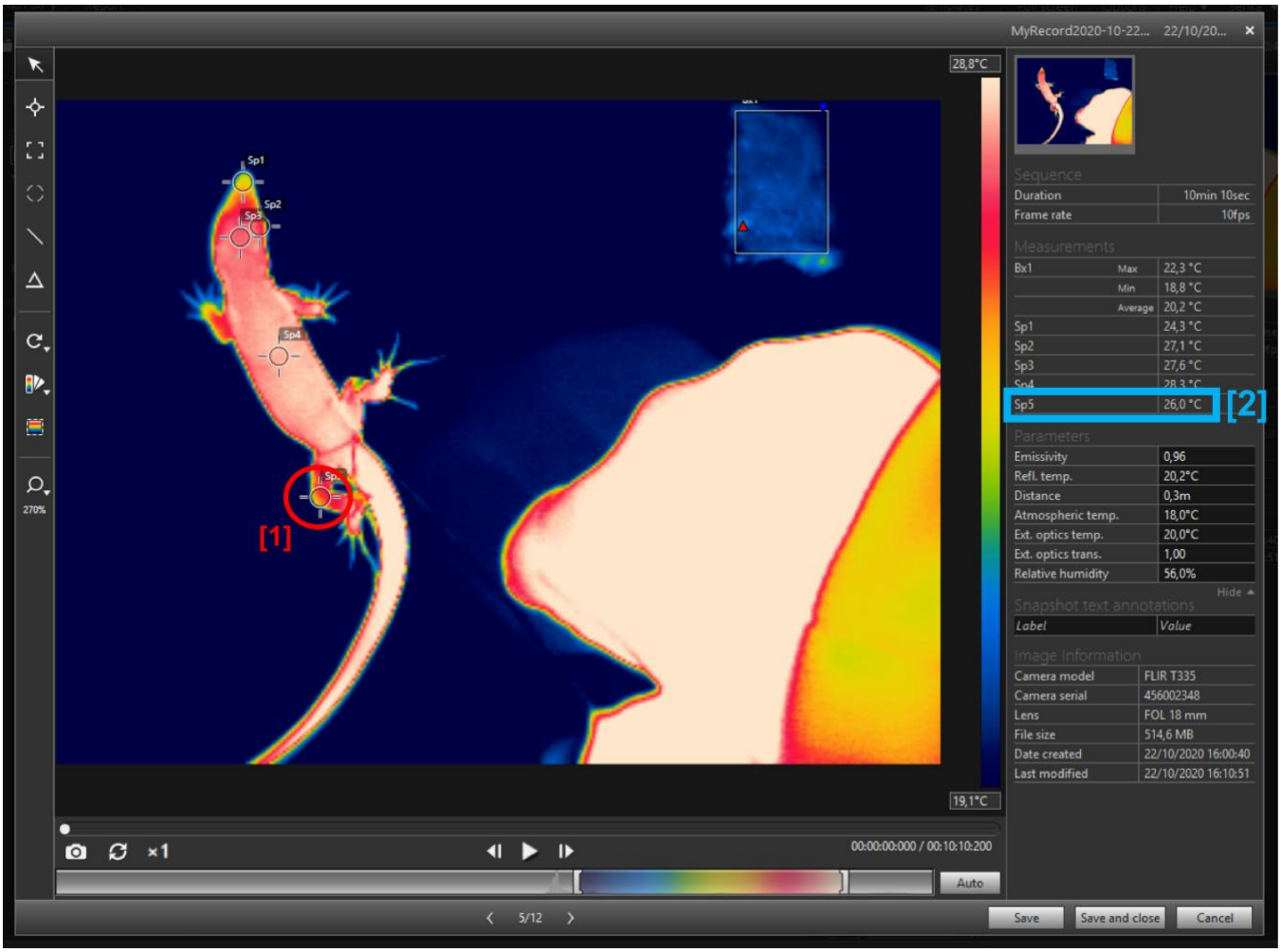
Record the temperature value for this spot [3] in the appropriate datasheet/database's column before moving to the next frame.



The 5th measurement will be of the **dorsum**. To do so a spot must be placed in the middle of the animal's dorsum [1]. Again, it is important to note if the animal is bending and/or tilting its body and take that into consideration when estimating the central point in the dorsum.

As with other measurements, if unhappy with the placement, delete the spot (select the spot + press *Delete*) and try again, **do <u>not</u> try to adjust the location of the dropped pin**.

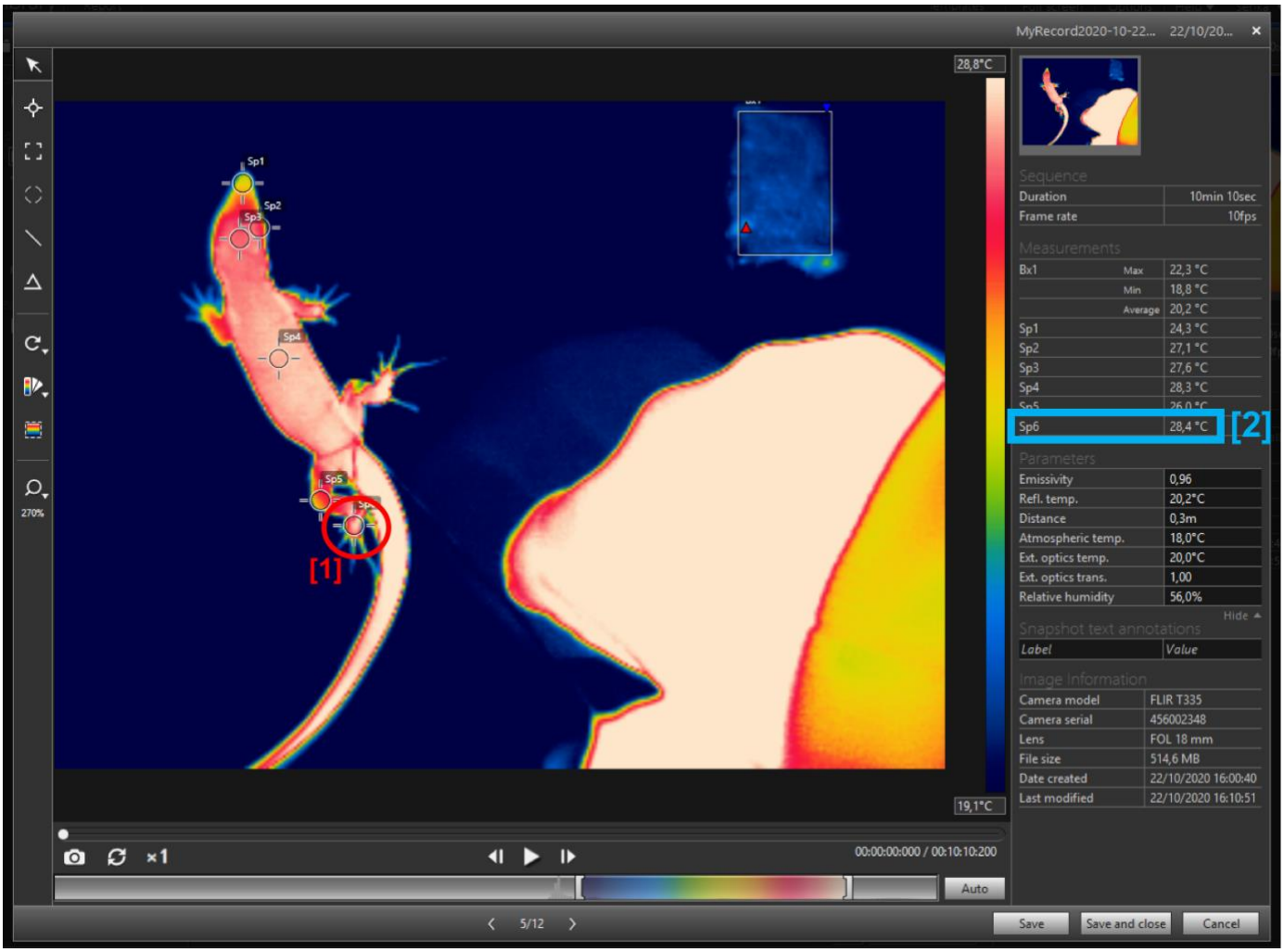
Record the temperature value for this spot [2] in the appropriate datasheet/database's column <u>before moving to the next frame.</u>



The 6th measurement will be of the **LEFT leg**. To do so a spot must be placed on the **knee of the LEFT HIND (back) limb** [1]. This body part is often hidden, and, in such cases, the measure is simply skipped for that specific frame.

As with other measurements, if unhappy with the placement, delete the spot (select the spot + press *Delete*) and try again, **do <u>not</u> try to adjust the location of the dropped pin**.

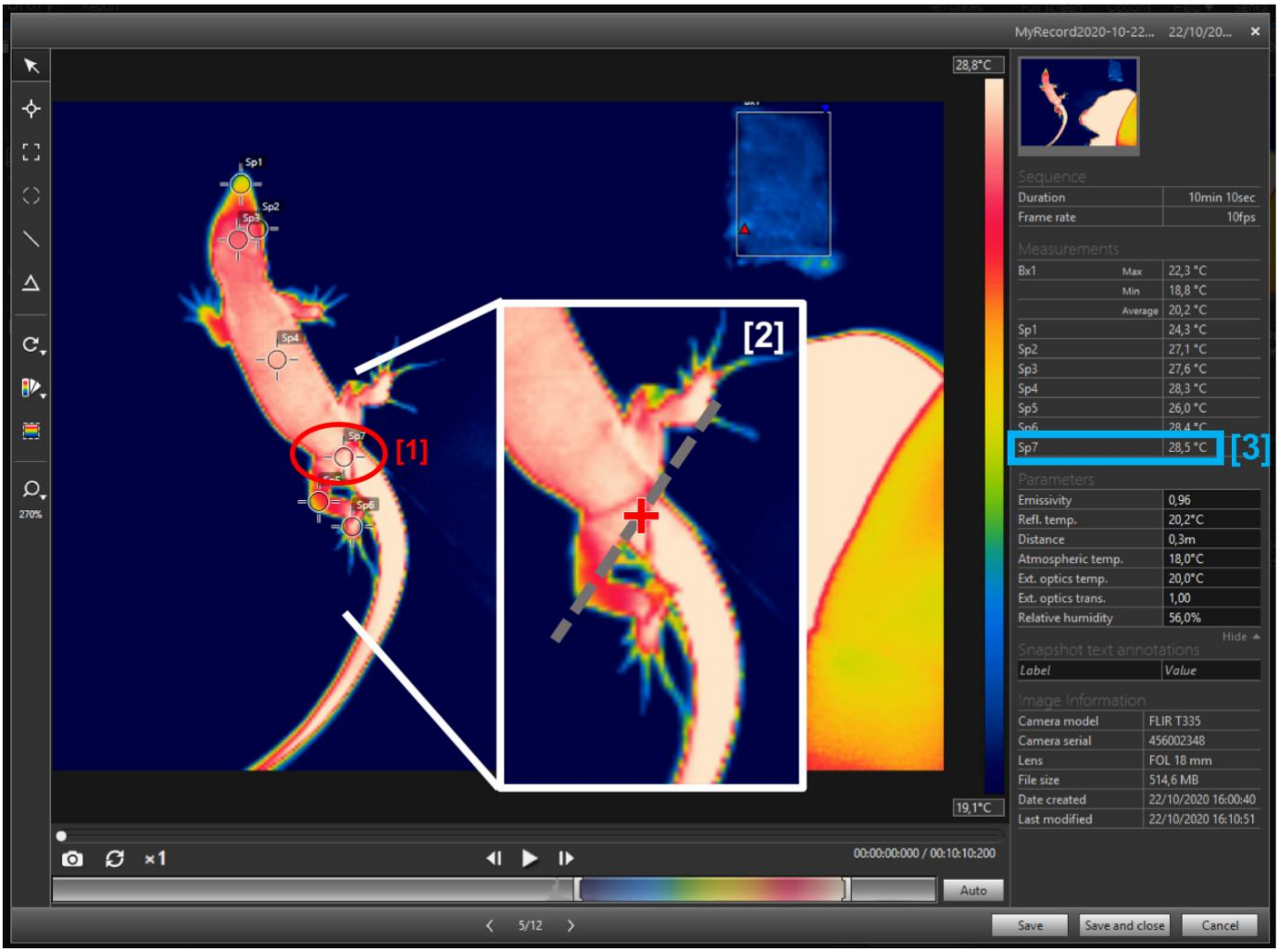
Record the temperature value for this spot [2] in the appropriate datasheet/database's column <u>before moving to the next frame.</u>



The 7th measurement will be of the **LEFT palm**. To do so a spot must be placed centrally on the **palm of the LEFT HIND (back) limb** [1]. This body part is often hidden and/or very out of focus, in such cases, the measure is simply skipped for that frame in particular.

As with other measurements, if unhappy with the placement, delete the spot (select the spot + press *Delete*) and try again, **do <u>not</u> try to adjust the location of the dropped pin**.

Record the temperature value for this spot [2] in the appropriate datasheet/database's column <u>before moving to the next frame</u>.



The 8th measurement will be of the **tail**. To do so a spot must be placed on the on the dorsal side of the tail, above where the cloaca would be found ventrally [1,2]. To locate the position, imagine a line connecting the end of the animal's thighs (dotted grey line in the zoomed box [2]) and place the pin at the midpoint of this imaginary line.

Caution must be taken when dropping the pin since the central location may be slightly obstructed by the presence of the animal's leash (visible in the example above). **The pin most NOT be placed on top of the string**. Instead the location of the pin may be adapted slightly (preferably along the anterior-posterior axis – i.e. back and forth, instead of side to side). If, however, most of the location is obstructed by the string, meaning the pin would have to be placed far from the ideal location, the measure of the tail for that frame must be skipped.

As with other measurements, if unhappy with the placement, delete the spot (select the spot + press *Delete*) and try again, **do <u>not</u> try to adjust the location of the dropped pin**.

Record the temperature value for this spot [3] in the appropriate datasheet/database's column <u>before moving to the next frame</u>.

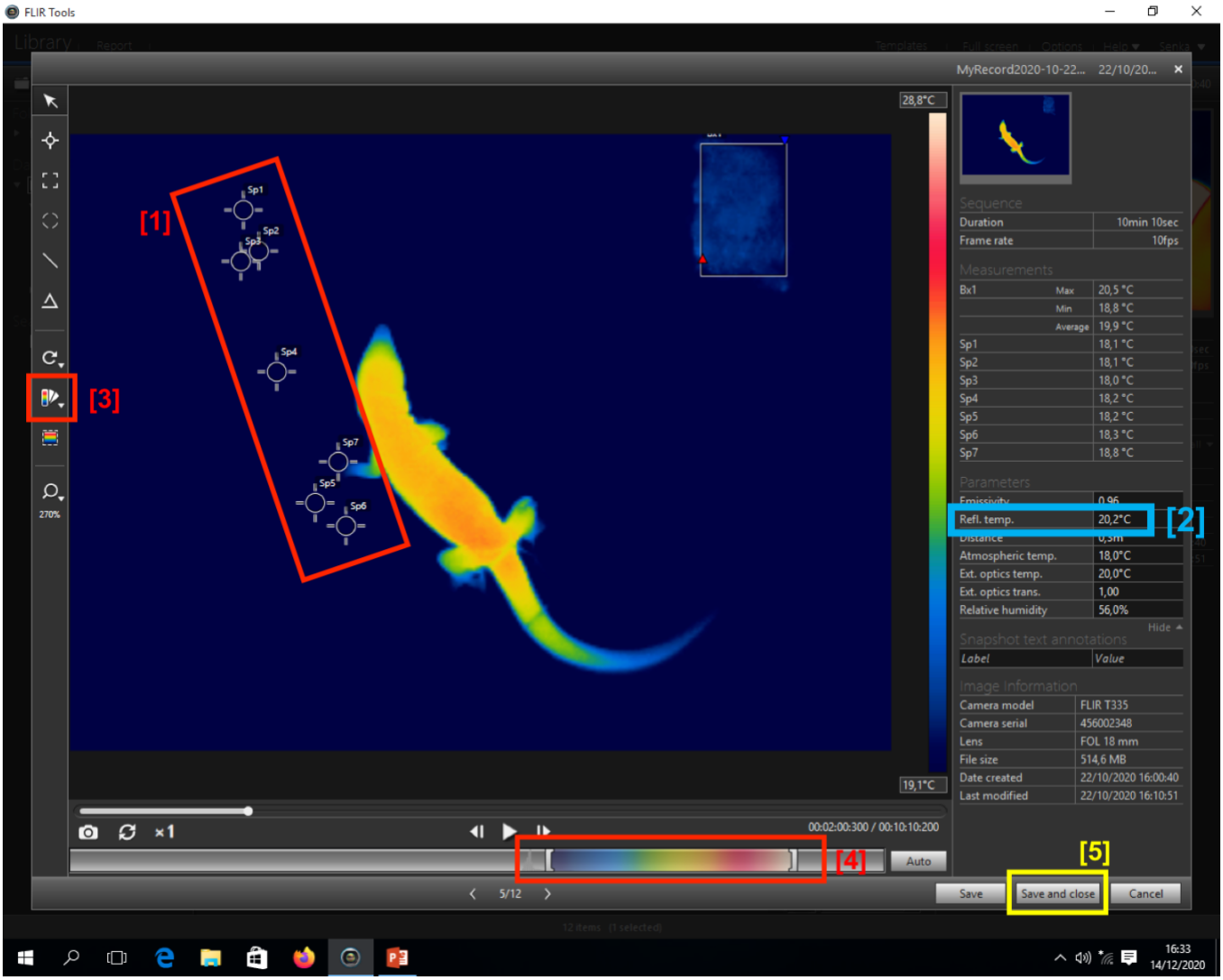
Then, if progressing to a further frame for analysis, then the steps after finding the desired frame are:

- Delete the measurement spots [1] (unless the subject has not moved **<u>at all</u>**);
- Check that the *Refl. temp.* is still the same (i.e equal to the *Average* of *Bx1)* or update it if needed [2]

It might also be necessary to:

- Adjust the *Palette* [3] or the colour/temperature range [4] to make the animal more visible;
- Adjust the size and or position of *Bx1* and/or *Bx2* (substrate temperature – not represented in this screenshot) if obstructed by the lizard

Once all these checks and adjustments are performed, then **repeat the previously described process to measure substrate, snout, right eye, left eye, head, dorsum, left leg, left palm and tail temperatures**.



Otherwise, if pausing or finishing the analysis, the video editor window may be closed by choosing “Save and close” [5] in the bottom right corner. This will save the location of the set measurement tools as well as the established parameters (global and local). This can be particularly useful if the analysis of such video will have to be finished at a later date.

Example Database for Recording the Data:

Video Reference: The name of the video file.

Movement: Whether the animal moved or not in the previous time slot.

Temperature of *[Body Part]*: The temperature of the different body parts, measured with the *Spotmeter* tool.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U
1	*(other variables, depending on study)*							VIDEO_REFERENCE	ELAPSED_TIME _(min:sec)	MOVE	T_SUBSTRATE	T_SNOUT	T_EYE_R	T_EYE_L	T_HEAD	T_DORSUM	T_LEG_L	T_PALM_L	T_TAIL	FOCUS	NOTES
2								MyRecord2020_22T...	00:20	No	18.1	32.3	30.8	32.8	31.4	27.9	29.2	31.3	27.9	Yes	
3								MyRecord2020_22T...	00:40	Yes	18.0	27.1	28.7	29.1	27.8	29.9	30.2	28.6	32.8	Yes	Animal over the aluminium square
4								MyRecord2020_22T...	01:00	No	18.2	32.8	30.0	31.6	29.3	30.9	30.3	28.2	32.7	No	
5								MyRecord2020_22T...	01:20	No	17.9	29.8	28.4	28.4	29.0	27.7	27.2	31.7	31.8	Yes	

Focus: Whether the subject of interest is perfectly in focus or not.

Notes: Any additional notes/remarks worth mentioning for the time slot.

Substrate Temperature: Temperature measured from a strip of electrical tape, of known emissivity, attached to the surface of the tank.

Elapsed Time: The time code associated for the frame of the measurement.

Summary of Steps:

1. Import video(s) into the FLIR Tools/ FLIR Tools+ library;
2. From the library, select the desired video to process and double click on it to prompt the video editor pop-up window;
3. Measure the [*Apparent*] *Reflective Temperature:*
    a. Place a *box measurement tool* creating a square/rectangle over the wrinkled aluminium reference;
    b. Avoid areas with abnormal hot/cold spots or obstructed by the animal;
    c. Right click on the set box and choose "Local parameters…" and set these for *Bx1 (*by ticking/selecting "Use local parameters" in the pop-up window) as*:*
        i. *Emissivity* = 1.00
        ii. *Distance* = 0
        iii. *Refl. temp* may be ignored or set to whatever value is pre-set in *Global Parameters*
4. Set the appropriate *Global Parameters* (press "Show all" to access all the parameters):
    a. *Emissivity* = 0.96
    b. *Distance* = 0.30 (or whichever distance was used between the subject and the camera)
    c. *Atmospheric temp.* = a defined <u>constant</u> measure of average ambient temperature during the recording process (18ºC)
    d. *Relative humidity* = a defined <u>constant</u> measure of average ambient relative humidity during the recording process (56% used from in-camera values)
    e. *Refl. temp.* = the *Average* temperature obtained from ***Bx1*** <u>for that frame</u>
5. Measure substrate/surface temperature by adding another *box measurement tool* ***(Bx2)*** but this time, over an area covered with black electrical tape of known emissivity ($\mathcal{E}$=0.96) making sure to:
    a. Avoid areas where the tape has wrinkles and/or overlaps;
    b. Choose an area less likely to be obstructed by the lizard throughout the video, to minimise the need to move Bx2;
    c. Choose an area with minimal reflections from outside thermal source (e.g. heat lamps);
    d. Avoid the edges of the tape when drawing the area of Bx2
6. The substrate/surface temperature will be the *Average* temperature of *Bx1*
7. Use the video controls to select the appropriate frame from which to obtain measurements;
8. To measure the temperature at the different body locations:
    a. Use the *Spotmeter* tool (one spot per location)
    b. Always use the cursor to select the precise location for the measurement before placing the *Spot.* If an error is made, delete the spot and try again. **Do NOT move an already placed spot to adjust it**.
    c. Measure the following body parts:
        i. Snout – find the mid-point between the two nostrils (intersection between the frontonasal and supranasal scales) avoiding the actual tip or the edges of the snout;

ii. Left and Right Eyes (on spot for each) – find the centre of the eyes, considering the head might be tilted and the view of the camera is from above (sub-optimal, considering the eyes are lateral). Adjusting the false-colour range and changing the palette, as well as relying on prior knowledge of the morphology of the animal will help to locate the eyes. If really not visible, ignore this measurement for that frame;

iii. Head – place the spot centrally and at the base of the head, where the occipital scale would be;

iv. Dorsum – place the spot in the middle of the animal's dorsum, considering its positioning/posturing and adjusting accordingly;

v. Left Leg – place the spot in the centre of the left, hind knee. If not visible, skip this measurement for that specific frame;

vi. Left Palm – place the spot centrally, in the palm of the left, hind limb. If not visible, skip this measurement for that specific frame;

vii. Tail – place the spot on the dorsal side of the tail, above where the cloaca would be. Imagine a line connecting the end of the animal's thighs and place the pin at its midpoint. The location may sometimes be obstructed by the animal's leash. If so, the location of the pin may be adapted slightly along the anterior-posterior axis. If not possible, the measure of the tail for that frame must be skipped.

d. Always record the temperature value for all the spots in the appropriate datasheet/database's column <u>before moving to the next frame.</u>

9. After adding the data to the database, **either**:

A) Progress to a further frame for analysis by:

- Finding the desired frame;
- Deleting the measurement spots (unless the subject has not moved **<u>at all</u>**);
- Checking that the *Refl. temp.* is still the same (i.e equal to the *Average* of *Bx1)* or update it if needed
- If needed:
    - Adjusting the *Palette* or the colour/temperature range;
    - Adjusting the size and or position of *Bx1* and/or *Bx2* if obstructed by the lizard.
- Going back to *step 8.*

OR

B) If pausing or finishing the analysis, close the video editor window by choosing "Save and close". This will save the location of the set measurement tools as well as the established parameters (global and local).

Acknowledgements:

A prior/pilot version of this pipeline was developed in conjunction with Gabriel Mochales-Riaño, Valéria Marques and Alexandra Telea, as part of a methodological study (in press) on lizard thermal exchange rates. The latter protocol was subsequently optimised, and here presented, by FMB. This has since been deployed by FMB and several other researchers who have provided important feedback to further improve this protocol. Hence, I thank Sara F. Nunes, Nikoletta M. Boskovits, Gesthimani-Myrto Roumelioti, Diana Silva and Luísa Lisboa for their input and/or validation of the protocol. I also thank Lekshmi B. Sreelatha for revising this protocol prior to submission, and Giulia Simbula for producing the labelled diagram of the lizard's head used throughout the protocol.

FMB was supported by consecutive PhD grants (SFRH/BD/147535/2019 and COVID/BD/153468/2023) from Fundação para a Ciência e Tecnologia (Portugal) during the development of this protocol.

References:

- Barroso, F. M., Carretero, M. A., Silva, F., & Sannolo, M. (2016). Assessing the reliability of thermography to infer internal body temperatures of lizards. *Journal of Thermal Biology*, 62, 90–96. https://doi.org/10.1016/j.jtherbio.2016.10.004
- Barroso, F. M., Riaño, G., Sannolo, M., Carretero, M. A., & Rato, C. (2020). Evidence from Tarentola mauritanica (Gekkota: Phyllodactylidae) helps validate thermography as a tool to infer internal body temperatures of lizards. *Journal of Thermal Biology*, 93, 102700. https://doi.org/10.1016/j.jtherbio.2020.102700
- Faye, E., Dangles, O., Pincebourde, S. (2016). Distance makes the difference in thermography for ecological studies. *Journal of Thermal Biology,* 56, 1–9.