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Monitoring fly feeding behavior and timing by beetle luciferase reporters V.2

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Monitoring *Drosophila* feeding behavior usually requires laborious procedures and custom-made equipment. This protocol describes how feeding behavior of individual flies carrying a beetle (e.g. firefly) luciferase gene can be conveniently monitored in real-time by measuring bioluminescence in 96-well microplates.

Drosophila flies expressing a beetle luciferase gene produce bioluminescence if fed with a food containing luciferin. Bioluminescence in flies appears within a minute and peaks within 2-3 minutes after a food ingestion. When refraining from eating or placed on a luciferin-free food, fly bioluminescence decays with about 0.3-1 hour half-life and essentially returns to the baseline after 4 hours. Naturally, under non-stressed conditions, flies eat sporadically and often make intervals of many hours between eating. This makes beetle luciferase bioluminescence a very convenient system to monitor fly feeding timing and, to a considerable extent, food intake in real time for many days (see the Abstract Figure). In this protocol I describe one of the possible procedures to monitor fly feeding using commonly available plate readers, outline a historical background on recording bioluminescence in live flies to study circadian gene expression, illustrate several examples of feeding behaviors that can be analyzed by this procedure and discuss some potential applications.

Bioluminescence signal in live freely moving flies is usually overwhelmingly dominated by fly feeding and movement. This makes firefly luciferase reporters poorly suitable to report changes in gene expression or other intracellular parameters in live flies despite their historically popular application for this purpose. Some approaches to address this problem are also discussed.

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INTRODUCTION

Beetle luciferases catalyze a bioluminescent reaction by oxidizing D-luciferin with molecular oxygen ([Carrasco-Lopez et al, 2020](#)). ATP is required as a second substrate to first convert D-luciferin into the activated luciferyl-adenylate form. Luciferases are widely used as reporter genes, most commonly via lytic assays of cell cultures and tissues. D-luciferin is relatively stable in solution for several days, so the beetle luciferase reaction can also be monitored in real time in live cell and tissue cultures for many days. Bioluminescence imaging is often used to track tumor development in live mice if cancer cells are labeled with a luciferase. When injected into mice, D-luciferin is relatively quickly eliminated from the bloodstream (~30 min half-life).

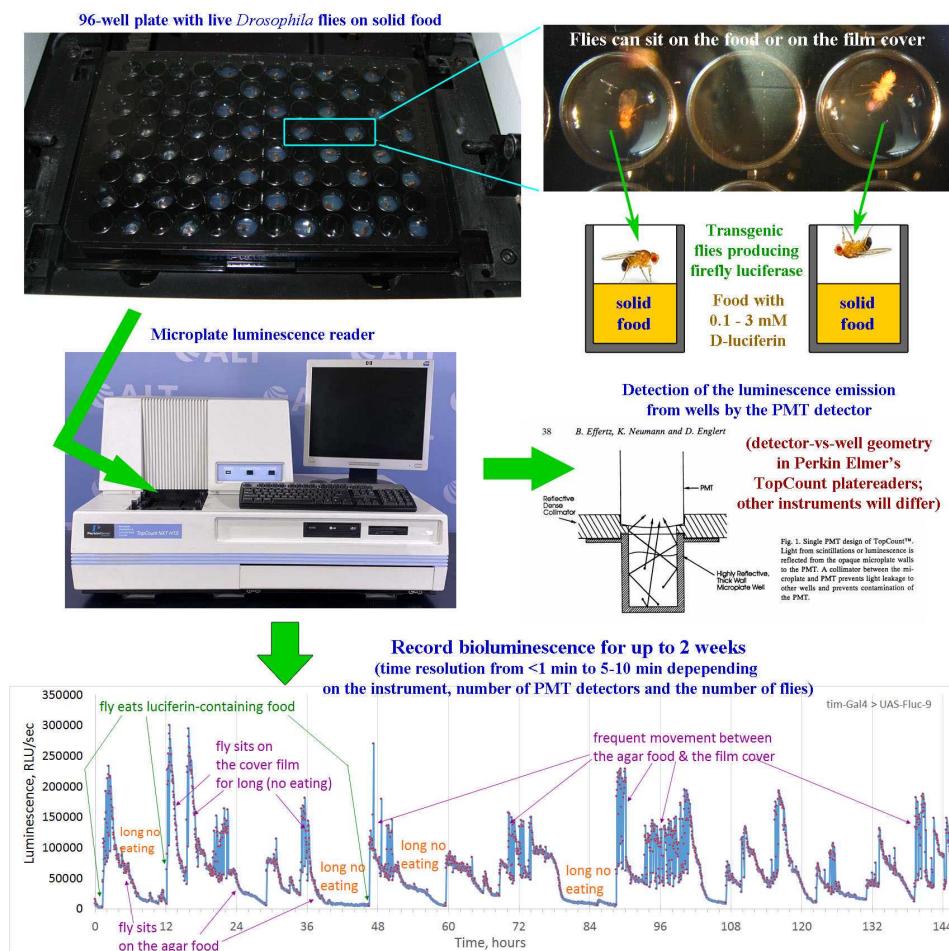


Fig. 1. Experimental workflow to monitor feeding of luciferase-expressing flies by bioluminescence recordings.

Monitoring *Drosophila* feeding behavior usually requires laborious procedures and custom-made equipment ([Deshpande et al, 2014](#); [Ro et al, 2014](#)). The described method (Fig. 1) allows to conveniently and precisely monitor the timing of food intake (as well as general movement of flies between the two

horizontal surfaces within a well) for transgenic luciferase-containing flies as they freely move within wells of a single 96-well plate. While the provided well space is small, flies can still move around and behave relatively naturally (see the videos in Step 8). When the D-luciferin substrate reaches a beetle luciferase enzyme produced inside fly cells, bioluminescence is emitted which is detected by a luminescence platereader.

The method relies on the following aspects of *Drosophila* fly biology and behavior:

- luciferin quickly (within 1-2 min) enters the fly circulatory system (and reaches a luciferase enzyme in cells where it is produced) after an ingestion of D-luciferin containing food ([Itskov et al, 2014](#))
- it is relatively quickly removed from the circulatory system unless replenished in flies and in mice
- when flies do not ingest food or ingest food without luciferin, bioluminescence decays with about 0.3-1 hour half-life and essentially returns to the baseline after 4 hours
- under normal conditions flies eat sporadically, often with >1 h intervals; food pass-through time can be as short as 15-20 min ([Deshpande et al, 2014](#))
- intervals between eating can be up to many hours and vary quite a lot between individual flies and between different genetic backgrounds (e.g. see FLIC measurements in Fig. 2.3 in the PhD Thesis of Qi Zhang ([Zhang, 2016](#)))
- repetitive lateral plate movement which occurs cyclically many times during measurements in luminescence plate readers (to place wells being measured under one or more PMT detectors) doesn't seem to disturb flies much and affect their behavior

The bioluminescence intensity measured from a well with an individual fly varies essentially because of the two factors:

1. Gradual signal decay due to luciferin excretion (0.3-1 hour half-time depending on a tissue) and an increase if new food is ingested
2. An abrupt 2-fold or higher change when a fly moves from the well bottom onto the film covering the well (since it is closer to the light detector and the ventral fly side generally absorbs less emitted light).

As long as the sampling rate of the recording is at least 5-10 minutes, these events can be clearly and easily discriminated in the recorded bioluminescence curves (Fig. 1).

This method can be used to:

- monitor feeding behavior *per se* under different conditions (e.g. how it depends on food types or genotypes; to look at circadian or other temporal questions)
- to simply detect when flies have ingested food for the first time or have refrained from eating after that (e.g. in experiments involving fly fasting or when flies need to consume various drugs and effectors in food which can be followed by subsequent experimental steps such as looking how the behavior or other parameters have changed).

HISTORICAL BACKGROUND and WARNINGS

The described protocol is based on a technique developed by groups investigating circadian gene expression ([Stanewsky, 2006](#); [Yu & Hardin, 2007](#)) to monitor circadian luciferase transcriptional reporters in live flies. Many useful experimental details on fly and plate preparation are described in these two referenced papers. This protocol can serve as an additional visual guide to prepare flies for this kind of experiments as well but, as can be seen from examples, extreme care should be taken not to interpret various feeding artifacts as intrinsic activity of these luciferase reporters.

In the current described method flies are recorded in a single plate at high temporal resolution to obtain detailed bioluminescence traces from individual flies (1-5 min resolution). In the referenced technique multiple plates are recorded in succession (by cyclical loading from a vertical stacker) with a low temporal resolution (30-60 min between measurements) and data from many flies (e.g. n=20-100) is averaged out without analyzing individual flies.

In general, bioluminescence of firefly luciferase reporters (typically, reporting gene transcription) can be monitored for many days in cell cultures ([Yamazaki & Takahashi, 2005](#)), brain slices ([Welsh & Noguchi, 2012](#)) and in detached fly organs and tissues ([Glaser & Stanewsky, 2005](#)). This approach was particularly popular in groups studying circadian gene expression. In all those cases cells are subject to a relatively constant (or consistently slowly decaying) level of luciferin in the culture media. This allows to precisely monitor genuine changes in luciferase activity which reflect daily changes in gene expression driven by promoters being studied.

As a rule of thumb, the change (amplitude) of luciferase activity is usually less than 2-fold for most circadian reporters ([Glaser & Stanewsky, 2005](#)) and can be up to around 3 for the best "artificial" reporter vectors in mammalian cells like Bmal1-luc (see the Fig. 2 below).

However, dramatic differences in luciferin concentration available to luciferase (thus affecting its detected activity) can become an issue in live animals where luciferin is relatively quickly excreted from the systemic circulation whether it is delivered by an injection or with food.

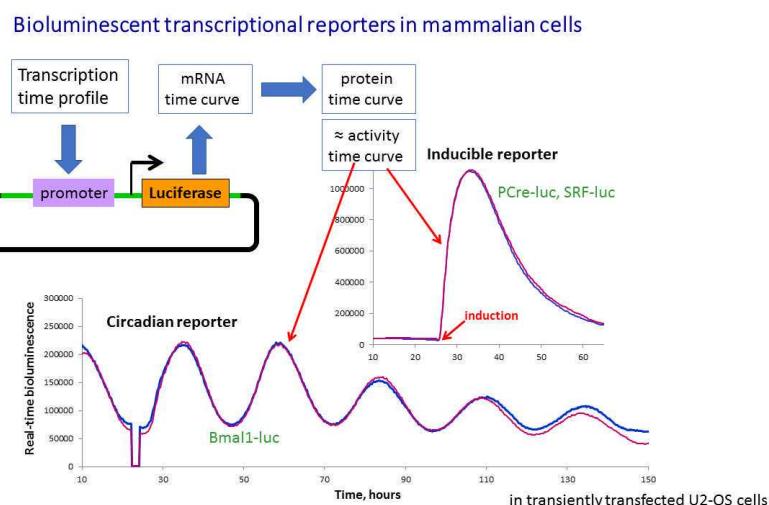


Fig. 2. Example of circadian (Bmal1-luc) and inducible (CREB-luc) luc2 luciferase reporters in mammalian cells (U2-OS cell culture).

Measurements of bioluminescent transcriptional reporters in live flies are orthogonal to monitoring feeding as it assumes and requires flies to eat continuously and uniformly, so that there is no change in luciferase signal due to feeding patterns which is otherwise dramatic (up to 10-fold and more) under more natural conditions as illustrated in this method (Fig. 1).

To some extent, the use of plate stackers and additional plastic domes inside wells indeed forces flies to eat more frequently but even then they usually still show feeding patterns: either more continuous regions with discrete gaps or, quite commonly, two continuous peaks during the 24 h with maximums around the middle of day and night and a more than 10-fold decrease of activity between these peaks (see Fig. 12, 13 and the Note 4 in the Steps section). This "two 12 hour peaks" pattern was reported for individual flies in early papers on this circadian technique where bioluminescence amplitude changes 10-fold or more in a circadian manner (see Fig. 6A in [Plautz et al \(1997\)](#), Fig. 2A in [Brandes et al \(1996\)](#)). This 12-hour pattern generally persists into many averaged out resultant curves and due to averaging the amplitude decreases to about 2-fold thus resembling typical circadian reporters (e.g. see Fig. 7 in [McDonald et al, 2001](#)) and less pronounced in Fig. 2 in ([Johnstone et al, 2022](#))). These 12 h rhythms are

commonly interpreted as a gene expression in many publications using this technique. However, Ralf Stanewsky correctly ascribed them to a some kind of behavioral artifact that still depends on circadian rhythms ([Stanewsky et al, 1997](#)) but it was not clear at that time that this is a feeding activity.

Overall, it seems that for most fly genotypes the feeding rhythms in bioluminescence cannot be really and reliably suppressed, so any luciferase reporters in live flies require very rigorous controls to show that any observed bioluminescence rhythms are not due to feeding or movement behavior.

This need for the rigorous verification by independent methods generally makes the use of luciferase transcriptional reporters in live flies unreliable as an independent method on its own (e.g. to screen for gene expression phenotypes). Some fly genotypes, however, may be more susceptible to showing continuous feeding under such conditions and, if properly controlled, can be used to monitor gene expression reporters. For example, XLG-luc flies seem to produce similar circadian bioluminescence rhythms both in separated fly organs in a liquid culture (where feeding timing is not an issue) and in live moving flies ([Glaser & Stanewsky, 2005](#)).

Note 1. Potential approaches to make luciferase reporters in live flies reliably work to track transcription and other intracellular events.

1) The movement of flies from the food to the film results in an abrupt increase in activity (Fig. 1) compared to gradual changes due to luciferin excretion or gene transcription. This effect obviously affects the tracking of feeding as well.

As long as flies are recorded individually and with at least 5-10 min sampling interval, these events can be marked and then adjusted for by an appropriate numerical analysis. Reference coefficients can be obtained for a signal from a fly in a bottom and a top position in a given experimental setup for the subsequent use.

Placing several flies in the same well (e.g. see Video 2) is sometimes used in an attempt to average out movement and feeding but in the end it rather makes interpretation of results more confounding, and flies still can show more synchronous rather than random behavior as a group.

2) The effects of feeding (and usually movement as well) on the signal can be efficiently accounted for by the use of dual-color luciferase reporters.

This approach was successfully used to track transcription in liquid yeast cultures where the signal otherwise can show remarkable changes due to differences in oxygen levels during yeast growth ([Krishnamoorthy & Robertson, 2015](#); [Robertson & Johnson, 2011](#)).

In this approach the transcriptional reporter can drive, for example, a green emitting luciferase while the constitutive reference reporter can drive a red emitting luciferase. Then the bioluminescence should be recorded simultaneously (or close to that) from the same fly using green and red bandpass filters. The bandpass signals then can be numerically decomposed into individual reporter time curves, and the signal of the constitutive reporter can be used to account for the dominating feeding and movement signal component.

For an efficient signal decomposition, the two reporters should be optimized to have a similar light output and have as large spectral separation as possible. The bandpass filter ratios of individual reporters are then used to decompose the dual (bimodal) signal. The red bandpass signal component will always have some contribution from the green reporter (due to the long red tail of emission spectra). It is possible to have an appropriate bandpass filter and a green/red reporters combination that will not have a contribution from the red reporter in the "green" channel.

- The ideal and most efficient technical approach is to do dual readings *simultaneously* by splitting the same light beam and measuring it by two separate detectors. This way any effects of movement or other factors are minimal. However, such set-up is not available in most (if any) plate readers, so this will require building custom equipment.
- The next best thing is the filter switching recording mode (e.g. in Biotek HTX platereader) where readings are done *consecutively* from the same well.

Note 2. Potential effects of oxygen levels on firefly luciferase signal in live animals.

The papers on the use of firefly luciferase reporters in yeast shaking cultures ([Krishnamoorthy & Robertson, 2015](#); [Robertson & Johnson, 2011](#); [Robertson et al, 2008](#)) report that firefly luciferase signal starts to proportionally decline when the dissolved oxygen concentration decreases in the range ~117-106 µM (55-50% saturation) and then abruptly collapses close to zero below 90-100 µM. The luminescence signal is very low and relatively negligible at or below ~57 µM dissolved oxygen (see Fig. 1 in [Robertson & Johnson, 2011](#)).

50-57 µM corresponds to the 35-40 torr (mmHg) oxygen pressure at 37°C. Meanwhile, the typical oxygen levels in healthy *mammalian* tissues are 30-40 torr (compared to 146 torr at air saturation) ([Wilson, 2008](#)). These levels are in the range of the reported dramatic decrease of luciferase activity. Therefore, oxygen levels can be an important factor limiting luciferase activity in tissues of mice and other mammalian models. Conversely, firefly luciferase can serve as an oxygen sensor in yeast cultures ([Robertson et al, 2008](#)) and at least in some animal tissues.

While oxygen levels in fly tissues are not readily available, it is likely that in many cases they are in the same range (though, can potentially attain higher values due to the tracheal respiratory system) as in mammals, and can also influence luciferase activity.

Interestingly, the major theory on how bioluminescent beetles turn on and off their bioluminescence is by oxygen availability ([Trimmer et al, 2001](#)). The reported property of firefly luciferase to get "turned off" at still perfectly physiological oxygen levels indicate that such mechanism is nicely compatible with a healthy tissue physiology without a need for non-physiological hypoxia levels. Previously, an exotic mechanism was suggested ([Trimmer et al, 2001](#); [Aprille, 2004](#)) where oxygen-consuming mitochondria physically prevent a diffusion of oxygen to the light-emitting organelles in the cell interior considered relatively hypoxic.

Note 3. On the use of firefly luciferase as an ATP-sensor in live flies.

Since ATP is a substrate of firefly luciferase, this system is widely used in optimized enzymatic assays to determine ATP concentration in solution. Based on this, it may seem promising to use it for the same purpose in live cells. Currently there is a relative interest to use bioluminescence of stably expressed (GAL4 x UAS-luc) firefly luciferase as a sensor of changes in ATP concentrations in live *Drosophila* flies. It may even seem tempting to interpret some of the observed changes in bioluminescence shown here as changes in tissue ATP levels.

However, there are two major problems which make unlikely for such reporters used in live healthy flies to reflect considerable differences in ATP concentrations:

- 1) In healthy tissues ATP concentration is very tightly controlled and considered to be quite stable with a very little room for temporal variation - which is well discussed in the following references: ([Wilson, 2011](#); [Heller, 2011](#)). While a number of publications do report dramatic temporal changes of ATP in animals and cell cultures under healthy conditions, such variations usually appear to be non-biological and a likely artifact of inappropriate sample preparation which fails to preserve ATP before the assay.
- 2) The commonly used *Photinus pyralis* luciferase actually has a relatively weak response in the range of ATP concentrations (2-6 mM) typically encountered in healthy tissues.

Its Michaelis constant (K_m) for ATP in a buffer solution is about 170 μM ([Branchini et al, 2007](#); [Fujii et al, 2007](#)) which would make the response particularly weak. As an illustration, an already non-physiological change of ±1 mM (±50%) from 2 mM ATP would result in only +3% or -7% difference in luciferase signal. Luckily, the cytoplasmic microenvironment was reported to increase the K_m(ATP) to 2-2.5 mM ([Allue et al, 1996](#); [Rangaraju et al, 2014](#)). In this case a similar non-physiological [ATP] change would lead to -35% or +18% change, respectively. This would still be problematic to reliably discern in recordings from live flies.

In principle, luciferase variants with higher K_m(ATP) values (>1 mM in a buffer solution) would be more promising in this regard. On the other hand, beetle luciferases with 1-10 μM values of K_m(ATP) can serve as convenient essentially "ATP-independent" controls.

You will need:

1) Flies that produce a beetle luciferase in some of their tissues.

Unless you create your own transgenic flies, the most straightforward way is to use some of the available UAS-luc lines and cross them to suitable Gal4 drivers.

A number of UAS-luciferase lines are available from several research groups:

- a set of UAS-luciferase lines inserted in various attP sites created in the Norbert Perrimon lab (Harvard University, USA); The luciferase is a luc(+) variant of *P. pyralis* firefly luciferase ([Markstein et al, 2008](#)).
- UAS-"LitGTS-luciferase"-EGFP lines created in the Gregory Macleod lab (Florida Atlantic University, USA). The luciferase is a mutant version of *L. italica* luciferase fused to GFP.
- UASp-Pmat luciferase (codon optimized *Pyrocoelia matsumurai kumejimensis* firefly luciferase with several thermostabilizing mutations ([Ogoh et al, 2020](#)).

These UAS-luc lines result in quite low target protein expression levels with many specific drivers (e.g. MEF2-Gal4, IFM-Gal4, etc) when compared to UAS-EGFP fluorescent lines developed in the Rubin lab (e.g. pJFRC81-10XUAS-IVS-Syn21-GFP-p10 and pJFRC14-10XUAS-IVS-GFP-WPRE, [Pfeiffer et al. 2012](#)). The latter usually show higher and more similar target protein levels across different drivers. So ideally a development of similar beetle luciferase lines is desirable.

A number of circadian reporter flies (e.g. BG-luc) are available from circadian fly labs ([Stanewsky et al, 1997](#); [Sehádová et al, 2009](#)) which usually carry a WT *P. pyralis* luc gene targeted to peroxisomes ([Stanewsky et al, 1997](#)). However, in these flies the levels of luciferase itself are expected to change during the 24 h period in a circadian manner, so they are less optimal for feeding experiments.

If you are going to create new luciferase transgenic lines, I suggest to use thermostable beetle luciferase.

If you are going to create new luciferase transgenic lines, I suggest to use thermostable beetle luciferases (improved mutant variants of *P. pyralis* luc or the ones derived from other species) rather than the most commonly used luc(+) or luc2 *P. pyralis* luciferase genes. These versions have a low thermal stability at temperatures above 30°C which can be undesirable in some experimental conditions.

For example, luc2.3 ([GenBank: MZ090948.1](#)), mVenus2-luc2.3, mVenus2-luc2.3-Nluc ([GenBank: MZ351723.1](#)) and Ppe2a ([GenBank: MZ090952.1](#)) reporter genes can be convenient.

Many Gal4 drivers can be used as needed as long as they provide a high enough signal for your luminescence reader. Sensitive platereaders (like TopCount) can reliably record luminescence even from weak drivers or plain UAS-luc (100-1000 RLU in TopCount) but higher signal levels are more convenient ($>1\times10^5$ RLU with r4-Gal4 and $>1\times10^6$ RLU with tim-Gal4 drivers). The tim-Gal4 driver ([Bloomington stock # 7126](#)) provides the highest luciferase expression levels (and bioluminescence signals) among many broad expression drivers; in adult flies it drives a broad expression in most soft tissues (while in larvae - only in a very small brain region). The fat-body specific r4-Gal4 driver ([Bloomington stock # 33832](#)) is another convenient driver resulting in good signal levels.

Examples of expression patterns driven by different drivers visualized by UAS-cyto-GFP (10XUAS-IVS-Syn21-GFP-p10).

Note that the dorsal side of a fly is much less light permeable than the ventral side (in this case - for visualizing GFP fluorescence). This is also important when fly bioluminescence is recorded from the top: flies can sit on the food or on the film. In the latter case they are closer to the light detector and are oriented with their ventral side up.

GFP expression of tim-Gal4>UAS-cyto-GFP flies



Fig. 3. A) Tissue expression pattern of the tim-gal4 driver.

GFP expression of r4-Gal4>UAS-cyto-GFP flies (fat body driver)



Fig. 3. B) Tissue expression pattern of the r4-gal4 driver.

GFP expression of IFM-Gal4>UAS-cyto-GFP flies (subset of flight muscles)

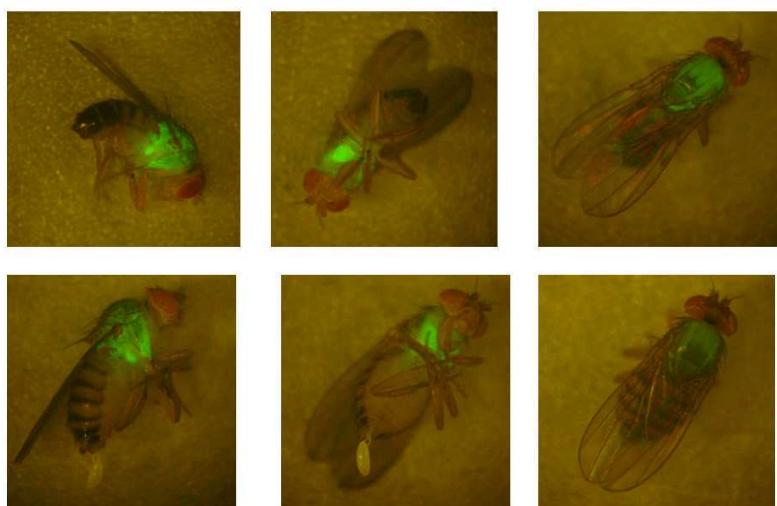


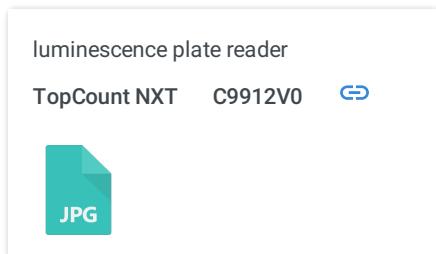
Fig. 3. C) Tissue expression pattern of the IFM-gal4 driver.
This driver results in a localized expression (intermediate flight muscles) in an adult tissue which is not critical for a fly survival ([Yun et al. 2014](#)).

2) A suitable luminescence plate reader.

Different versions of TopCount (Perkin Elmer, USA) are the most commonly used for fly recordings ([Tanenhaus et al. 2012](#)). TopCount NXT is the latest version; currently this product line is replaced by MicroBeta2 Microplate Counters by Perkin Elmer. These instruments are optimized for the highest sensitivity (PMT detectors are brought in a close proximity to wells ([Effertz et al. 1993](#)) and can have up to 12 PMT units, so even the whole 96-well plate can be read only in several minutes.

However, other plate readers should also be well suitable, e.g. CLARIOstar microplate reader ([Dag et al. 2019](#)), and may be even beneficial if they have additional useful features. For example, Synergy HTX (Bioteck, USA) has an anti-condensation mechanism (to prevent water condensation on a cover film), programmed shaking and excitation light capabilities (could be used to manipulate or study relevant fly behavior), bandpass filters. In principle, portable luminometers measuring single splittable wells (from dividable microplate well strips) can also be used but they will only be able to take measurements from a single fly.

The relative luminescence units (RLU) produced by a plate reader can be referenced to chemiluminescence of coelenterazine in the presence of cyclodextrins ([Koksharov, 2021](#)) or to chemiluminescence of luminol ([O'Kane & Lee, 2000](#)) to make the reported absolute RLU values transferable and comparable across different instruments and research groups.



3) 96-well microplates (black or white color)

OptiPlates (Perkin Elmer, USA) or other standard opaque 96-well plates can be used:

[OptiPlate-96 White Opaque 96-well Microplate Perkin](#)

[Elmer Catalog #6005290](#)

[OptiPlate-96 Black Opaque 96-well Microplate Perkin](#)

[Elmer Catalog #6005270](#)

- White plates result in a higher bioluminescence signal.
- Black plates absorb more of the emitted light by well walls resulting in lower signals but they also reduce a signal cross-talk between wells.

4) adhesive transparent plate covering films

a) the cheapest (and commonly used) variants are covered with an adhesive: they work fine but there is a small chance that a fly wing will stick to it during placement of unconscious flies into wells or if very little free volume is

left for a fly to move

Possible choices:

[☒ TopSeal-A PLUS Perkin](#)

[Elmer Catalog #6050185](#)

[☒ TempPlate® Sealing Film USA](#)

[Scientific Catalog #2921-0000](#)

b) more expensive variants have an encapsulated adhesive which is released only under pressure (so there is no chance that fly wings will stick to it)

Possible choices:

[☒ TempPlate RT Select qPCR Sealing Film USA](#)

[Scientific Catalog #2921-7800](#)

[☒ PlateSeal Optically Clear Polyolefin Film Thomas](#)

[Scientific Catalog #1150P97](#)

[☒ ThermalSeal RTS™ Sealing Films for qPCR, Storage & Crystallization Excel](#)

[Scientific Catalog #TSS-RTQ-100](#)

5) D-luciferin (free acid form, sodium or potassium salt - depending on your preference or experimental needs)

[☒ D-Luciferin, free acid Gold](#)

[Biotechnology Catalog # L-123-1](#)

[☒ D-Luciferin, Sodium Salt Gold](#)

[Biotechnology Catalog #LUCNA-1G](#)

[☒ D-Luciferin, Potassium Salt Gold](#)

[Biotechnology Catalog # LUCK-1G](#)

Prepare a 100 mM stock solution (pH~7) and aliquot. The solution can be stored frozen at -80°C for at least 6 months. If needed, the stock solution can be sterilized by filtration.

Sodium and potassium salts of luciferin can be dissolved in water directly. The free acid form (= luciferin at low pH) has very low solubility.

To dissolve the free acid form, put the powder in water (e.g. 250 mg in 8.25 ml H₂O), mix by a magnetic stirrer and slowly add a base (usually, 5-10 M NaOH) until luciferin is dissolved (pale yellow-green solution) which occurs at pH~6.4. 25 mM HEPES or MOPS can be added to the mix before the dissolution to provide a buffering capacity (otherwise pH easily rises above pH 7-8 (intense yellow-green solution) where luciferin is less stable). Adjust the pH to 6.4-7 and adjust the volume to obtain a 100 mM solution.

6) a fly food mix (most commonly - with agar) that is initially in a liquid form when hot and will solidify after dispensing into plate wells.

The most basic and simple composition is a gel containing 5% sucrose and 1% agar which is commonly used in circadian and sleep research on *Drosophila*. Healthy flies generally maintain normal activity and behavior on it for at least 2 weeks.

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Read the Guidelines and Materials sections.

Preparation of a 96-well plate with a luciferin-containing food

1 Prepare a hot fly food mix and keep it unsolidified by placing a tube with food into a 48-50°C water bath.

at 50 °C

- a) It can be a regular complex cooked fly food (such as [BDSC Cornmeal Food](#)).
- b) It can be various synthetic and semi-defined formulations with 1% agar.

5% sucrose, 1% agar mix is commonly used when studying fly circadian behavior and sleep ([Versteven et al 2020](#)). Healthy flies live on it and retain normal activity often for more than 2 weeks. (To some extent this seems to be dependent on microbes which colonize the food, live off sucrose and trace elements in this food then supplying some nutrition to flies.)

In case of synthetic and semi-defined formulations with 1% agar, dissolve the components in water, then add 1% agar and prepare a hot liquid mix by heating in a microwave. Heat sensitive components can be added when cooled down to 50-60°C.

2 While the liquid mix is kept in a 50°C water bath, add luciferin from a 100 mM stock to have a desired concentration (0.1 mM - 3 mM, or higher) and mix thoroughly. **at 50 °C**

[M]3 millimolar (mM) D-luciferin

3 mM luciferin is a commonly used concentration providing good signal levels.

[M]0.1 millimolar (mM) D-luciferin

0.1 mM luciferin leads to a 2-3-fold lower signal compared to 3 mM concentration which is not an issue in most cases. This very low concentration could be used as likely non-interfering with fly behaviors and to conserve the luciferin substrate. (Since luciferin has sodium or potassium as a counter-ion, these metal ions may affect the food taste to some extent at high luciferin concentrations if used in a simple food like 5% sucrose.)

Luciferin analogs such as Akalumine-HCl (cas # 1176235-08-7) or CycLuc1 (cas # 1247879-16-8) can also be used if needed. They are less soluble and much more expensive, so it is convenient to prepare food with a 0.1 mM concentration. UAS-luc flies (producing luc(+) *P. pyralis* luciferase) show about 10-fold lower signals with them compared to 0.1 mM D-luciferin. These substrates could be useful due to their red-shifted

light emission (e.g. for dual-color measurements or for a better tissue penetration), particularly if coupled to mutant luciferases designed to have high activity with them ([Iwano et al, 2018](#))



Flies in the 96-well plate on a food (5% sucrose / 1% agar) with different luciferin analogs (0.1 mM): D-luciferin (lanes 1-2; translucent color), CycLuc1 (lanes 3-4; green) and Akalumine-HCl (lanes 5-6; reddish). Lanes 7-8 contain 3 mM D-luciferin.

3 Dispense 100-300 μ l food into the wells of a 96-well plate.

Wait 20-30 min for food to solidify.  **Room temperature**

In experiments where a minimal signal cross-talk between wells is important, the luminescence carry-over between neighboring wells can be minimized by filling and using every other well for the fly recordings.



96-well plate filled with about 300 μ l food per well: either a regular fly food (yellow-brown) or 5% sucrose / 1% agar (translucent).



100 μ l food provide the highest amount of space for a fly to move inside the well and will increase the signal change when flies move between the food surface and the film cover.

Higher volumes of food can be beneficial during multi-day recordings since they will dry out slower. Higher food volumes are also used in circadian protocols to minimize fly movements as much as possible ([Stanewsky, 2006](#)).

In some of the older papers there is a suggestion to first place 200-250 µl of luciferin-free food and then, after it solidifies, top it up with 50-100 µl of luciferin-containing food "to save expensive substrate" ([Stanewsky, 2006](#)). However, the luciferin from the top layer will get equilibrated [by diffusion throughout the whole volume after several hours](#), so this additional step is unnecessary and will result in lower luciferin concentrations than intended.

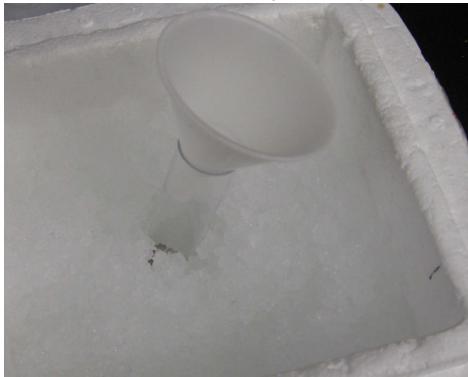
Placing flies into 96-well plates

4

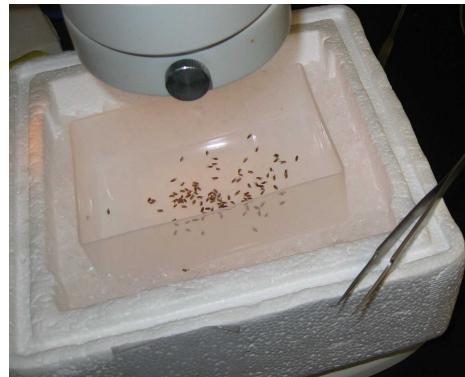
Anesthetize flies by cooling them on ice or by using a CO₂ pad. The CO₂ pads are the most common method used in fly labs while cooling is the simplest. "In addition, it [cooling] is the only method which will not affect fly neurology, therefore behavior studies may begin after the flies have warmed up sufficiently." (See [the manual from the Berg Lab](#))



Flies were anesthetized by cooling on ice.



Flies are immobilized by cooling on ice. Drop the flies into the ice-cooled tube through a funnel.



Cold anesthetized flies are placed on a flat ice-cooled vessel for handling.

Note: an actual temperature on ice cooled flat plastic surfaces is a few degrees above 0°C. Flies kept at a room temperature (20-25°C) are rendered immobilized on such surfaces. However, it should be mentioned that flies kept at 5-16°C for at least a few hours undergo some cold adaptation and usually have an ability to slowly walk on such surfaces.

5 As anesthetized flies are laying on the cooled box (plate) or on a CO₂ pad, take a chosen fly by the wing with tweezers (or take it by a small brush) and put it into a plate well.

The transfer of all flies should be done quickly so that flies won't resume moving and escape from uncovered plate wells.

6 Load flies into wells of a 96-well plate and seal the plate with an adhesive plate sealing film. Several approaches can be used:

a) After quickly loading the whole plate, carefully cover the plate with a press-on transparent adhesive film.

Care should be taken to avoid static electricity when detaching the film from its backing liner and handling.

Otherwise, flies can get pulled out from wells by a static charge.

Firmly attach the adhesive film by applying pressure across the whole surface either by fingers or by a plastic sealing pad. The use of a sealing pad is more important in case of films that have an encapsulated adhesive which is released only under pressure.



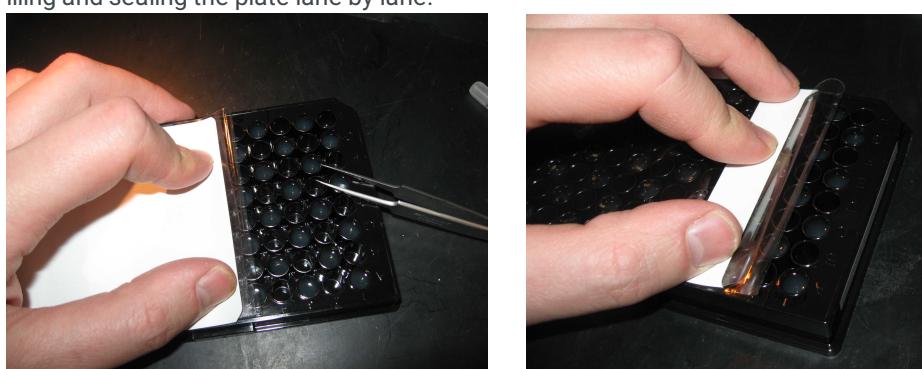
Flies are placed into a 96-well plate (every other well) and covered with an adhesive film.



b) Alternatively, the flies can be loaded lane by lane gradually sealing each lane after flies are loaded. In this case, there is less risk than flies will start moving and leave the wells. Also, the problem of static electricity is less likely to appear.



Filling and sealing the plate lane by lane:



c) The whole plate can be cooled and kept on ice to keep flies anesthetized.

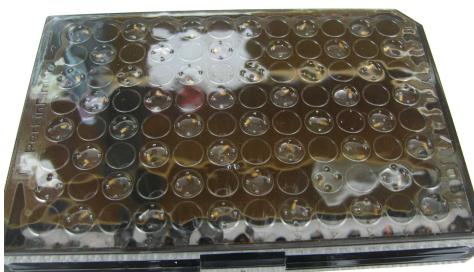
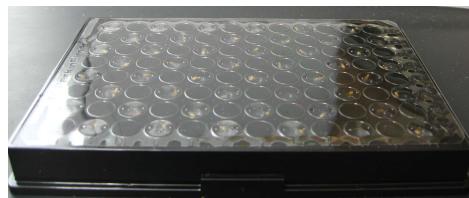
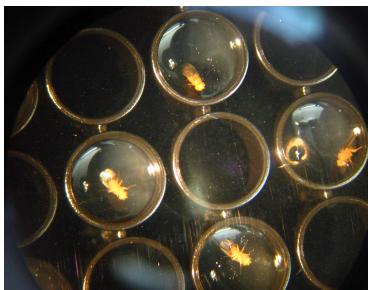
When using adhesive films covered with an unprotected adhesive, care should be taken that flies don't stick to it with their wings during placement. This can happen due to an attraction by a static charge or if the free volume above food is very small. Flies trapped this way are often not able to release themselves.

Otherwise, such films are perfectly compatible with flies living in wells for 1-2 weeks. The commonly used sealing film of this type is TopSeal-A Plus (Perkin Elmer, USA) ([Stanewsky, 2006](#); [Yu & Hardin, 2007](#))

- 7 Poke 2-4 small holes in a film above each well for ventilation (for example, by a tip of a syringe needle). This is necessary to provide a fly with air. Otherwise, a fly will run out of oxygen in a couple of hours and die.



Ventilation holes in adhesive film above wells:



- 8 Flies move in the wells within the free space between the food layer and the transparent film cover. While the provided well space is small, it is sufficient to move around and flies behave relatively naturally. In terms of spacing, 3-4 flies can easily be kept in a single well if desired (but this would rarely make experimental sense in feeding experiments). Overall, this is likely not dramatically different from common crowded conditions often occurring in fly breeding tubes.



Videos of flies moving in microplate wells.

1) Video 1: single flies in wells

2) Video 2: 3 flies in some of the wells:

Record the plate luminescence in the platereader

- 9 1. Program the luminescence plate reader for continuous measurements with at least one measurement per fly every 5-10 min. Typically, a signal is integrated during 15-30 sec for each well (fly); this can be adjusted depending on a signal strength.

- Load the plate into the luminescence plate reader and start measurements. The experiment can be conducted for many days.

Details of suitable software settings for TopCount instruments (Perkin Elmer, USA) were described in methods papers on using bioluminescent circadian reporters in live flies ([Stanewsky, 2006](#); [Yu & Hardin, 2007](#)).

- Flies inside the plate reader are in a constant darkness.
- If light/dark cycles are needed, most readers can be programmed to remove the plate from the reading chamber for a specified time period before the next measurement. Then for the time outside the platereader the flies will be subject to the outside light/dark conditions.

- Flies generally seem to quickly accustom to regular horizontal plate movements which occur during measurements or during loading/unloading plate into the reader. They do not seem to change their position inside wells or get agitated in response to these movements.
- in an ideal technical set-up the plate should be static while the detectors would move but this is not available in typical plate readers
- it is crucial to use a single plate and not to use vertical stacker columns to load/unload multiple or even a single plate. As shown in examples, the use of a stacker disturbs flies and causes them to eat more frequently (likely, by abrupt shakes and taps during loading a plate into a stacker). This effect should be avoided unless desired for a specific purpose.

The TopCount NXT instrument (Perkin Elmer, USA) equipped with 12 PMT units permits very fast rates of continuous plate measurements, for example:

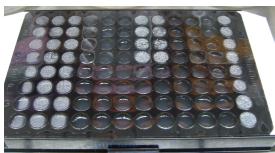
- all 96 wells: measurement every 3 min (15 sec integration time per well)
- every other well (48 wells): measurement every 2.5 min (15 sec integration time per well)
- 12 wells only: measurement every 18 sec (7 sec integration time per well)

Some plate readers may have features useful for certain kind of experiments. For example, programmed plate shaking, delivery of excitation or transmission light to wells, selection of excitation and emission wavelengths, etc (e.g. Bioteck plate readers).

Water condensation on the inside of the film sometimes becomes a problem, especially if the plate is removed frequently from the chamber. This happens when the film is consistently colder than the plate.



Heavy water condensation inside some of the wells



No water condensation in sealed wells with high media osmolarity compared to a pronounced condensation in wells with regular agar media.

This can be addressed by several approaches:

- use an anti-condensation feature if the plate reader has it (it keeps the temperature above the plate slightly higher)
- poke more holes (e.g. 4 instead of 2)
- try to tweak the cooling or heating systems of your platereader
- tweak the temperature and humidity outside the platereader
- use food with a higher osmolarity (e.g. 30% sucrose, 15% glucose, 13% xylitol, addition of NaCl, combinations thereof, etc) which reduces the vapor pressure inside wells

Process, plot and/or analyze the data

- 10 The obtained readings data can be further processed to obtain "luminescence - time" curves and analyzed by different approaches depending on the purpose of experiments.

Flies are being recorded in real time, so in relevant experiments flies that have eaten or refrained from eating for a defined time can be identified and collected for further processing.



A bioluminescence curve from an individual fly reflecting its feeding activity.

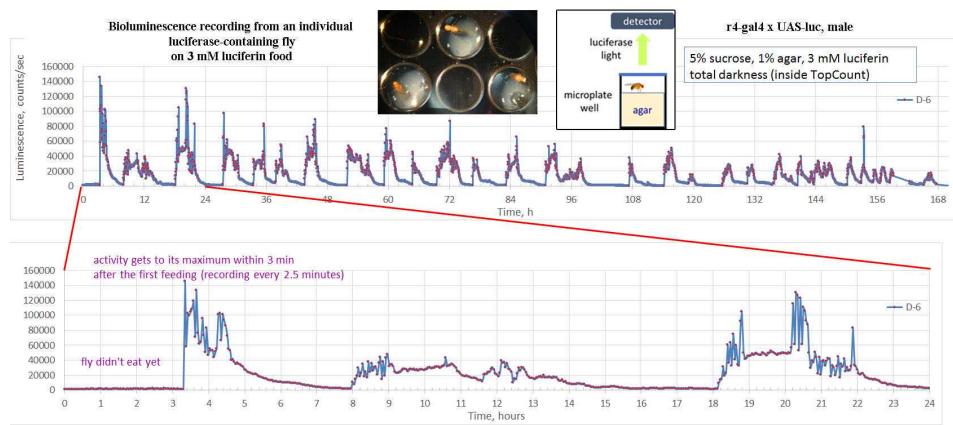


Fig. 4. A) A bioluminescence curve from an individual fly reflecting its feeding activity on 5% sucrose, 1% agar, 3 mM luciferin. Occasional spikes are due to movement of the fly to the film surface.

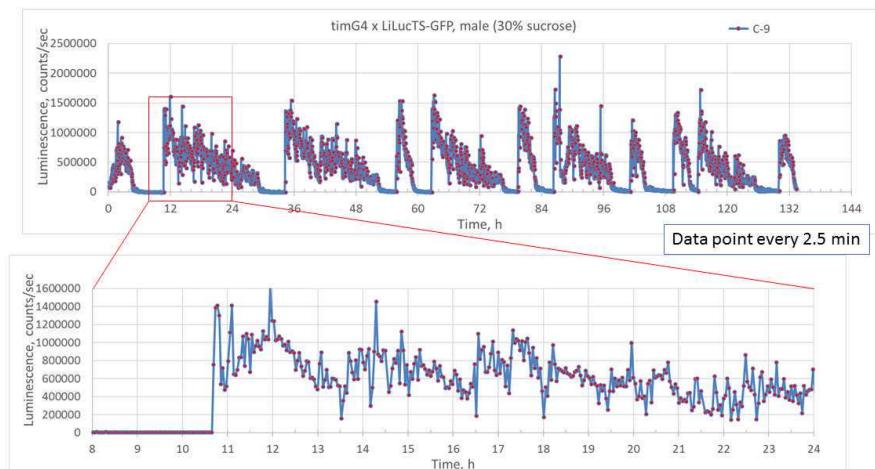


Fig. 4. B) A bioluminescence curve from an individual fly on 30% sucrose, 1% agar, 3 mM luciferin. Lower panel is an expanded view of the hours 8-24.

Examples of bioluminescence patterns due to feeding

11 This section lists examples of representative bioluminescence patterns reflecting different feeding behaviors of individual flies at various experimental conditions.

They serve to illustrate:

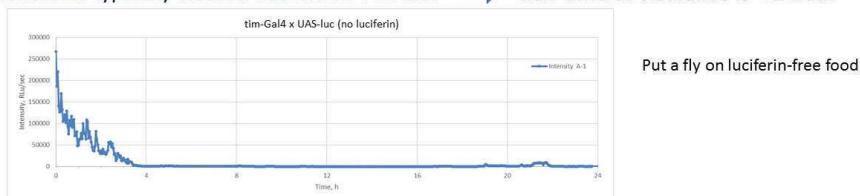
- the principles and assumptions of the method
- different feeding behaviors depending on a food type, experimental conditions and fly differences



Bioluminescence returns close to the baseline after 4 hours if luciferin-fed flies are placed on luciferin-free food or simply refrain from eating.

Luciferin clearance from flies pre-fed with 15 mM luciferin:
put them on luciferin-free or to 3 mM luciferin food → record in plate reader

luciferin is typically cleared out within 4 hours: half-time of clearance is < 1 hour



On *luciferin food* the signal can similarly disappear within 4 h (but comes back later)



10

Fig. 5.



Bioluminescence decays with about 1 hour half-life in case of the tim-Gal4 driver (Fig. 6) at various luciferin concentrations when flies refrain from eating.

Depending on a tissue driver, the half-life of the signal decay can be shorter: for example, about 0.4-0.5 h for r4-gal4 and 0.1-0.15 h for MEF2-gal4 (not shown). This likely reflects differences in luciferin pharmacokinetics for different tissues.

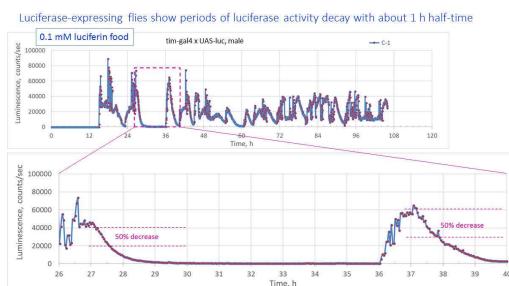


Fig. 6. A) Examples of bioluminescence decay on 0.1 mM luciferin.

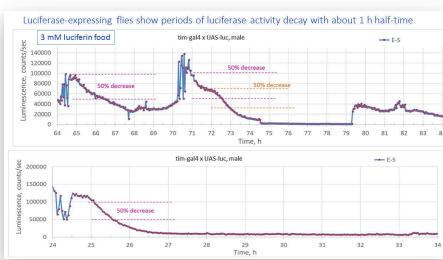


Fig. 6. B) Examples of bioluminescence decay on 3 mM luciferin.

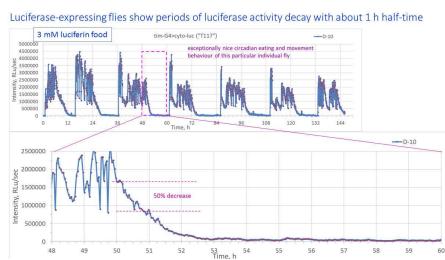


Fig. 6. C) Examples of bioluminescence decay on 3 mM luciferin.

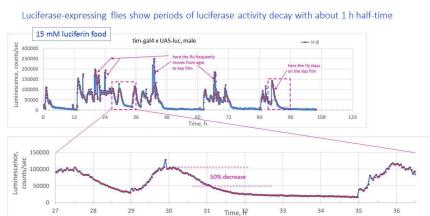


Fig. 6. D) Examples of bioluminescence decay on 15 mM luciferin.

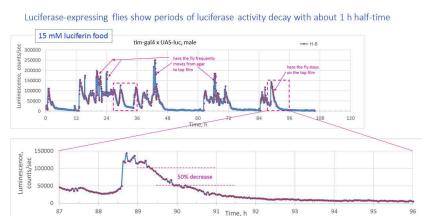


Fig. 6. E) Examples of bioluminescence decay on 15 mM luciferin.



Feeding behavior is very similar on regular (complex) fly food and on the simple 5% sucrose, 1% agar mix.

Patterns of bioluminescence on 5% sucrose / 1% agar are similar to those on the regular complex fly food → the behavior seems natural

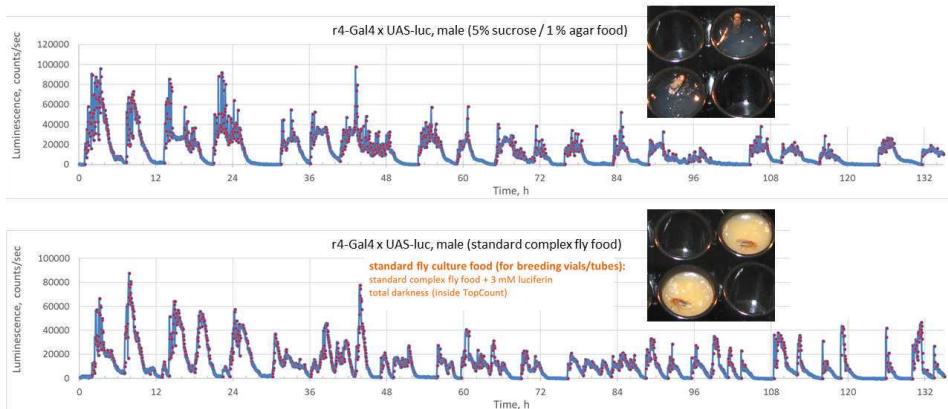


Fig. 7. Bioluminescence patterns of two representative flies: the first one - on 5% sucrose, 1% agar, 3 mM luciferin; the second one - on regular fly food with 3 mM luciferin.



Interpretation of bioluminescence curves in terms of fly feeding and movement.

The bioluminescence intensity measured from a well with an individual fly varies essentially because of the two factors:

1. Gradual signal decay due to luciferin excretion (~ 0.3-1 hour half-time) and an increase if new food is ingested
2. An abrupt 2-fold or higher change when a fly moves from the well bottom onto the film covering the well (since it is closer to the light detector and the ventral fly side generally absorbs less emitted light).

As long as the sampling rate of the recording is at least 5-10 minutes, these events can be clearly and easily discriminated in the recorded bioluminescence curves.

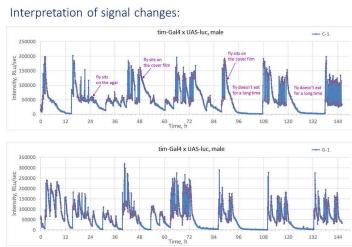


Fig. 8. A)

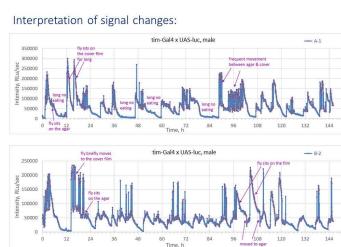


Fig. 8. B)

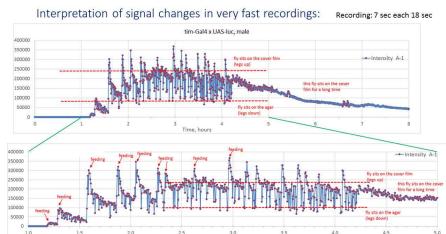


Fig. 8. C)



The method nicely shows when a fly starts eating.

When placed in wells (5% sucrose, 1% agar), flies can start eating right away or can voluntarily refrain from eating for >12 hours.

After placing in 96w plate, flies start to eat from immediately to >14 hours

Flies (LH2-free) are placed on luciferin food (illustrative signal curves):

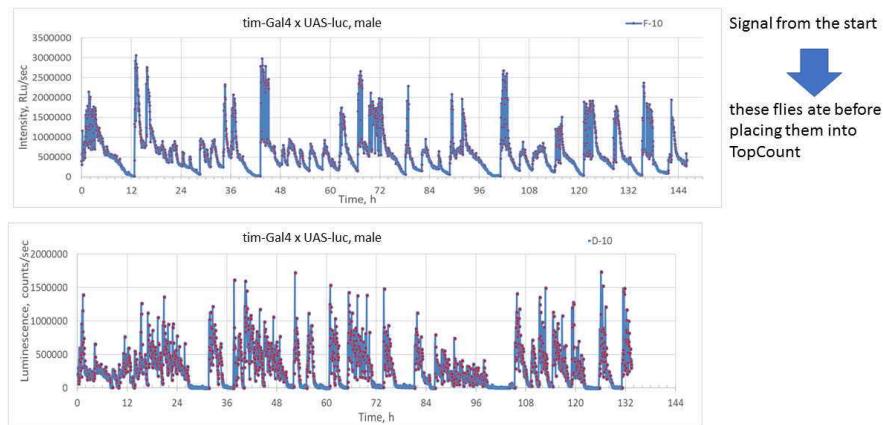


Fig. 9. A) Representative flies that have started to eat immediately after being placed into the plate (5% sucrose, 1% agar, 3 mM luciferin).

After placing in 96w plate, flies start to eat from immediately to >14 hours

Flies (LH₂-free) are placed on luciferin food (illustrative signal curves):

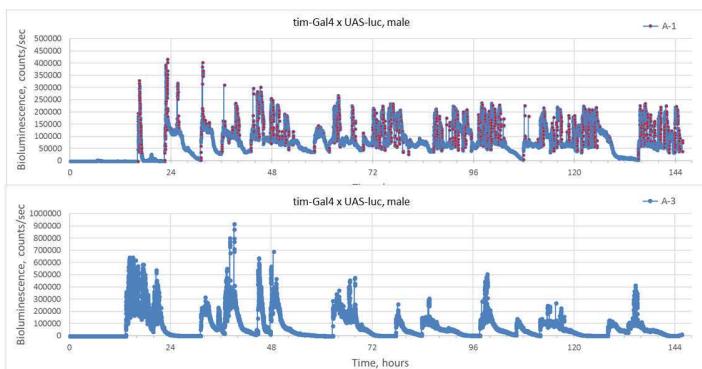


Fig. 9. B) Representative flies that have started to eat more than 12 hours after being placed into the plate (5% sucrose, 1% agar, 3 mM luciferin).

After placing in 96w plate, flies start to eat from immediately to >14 hours

Flies (LH₂-free) are placed on luciferin food (illustrative signal curves):

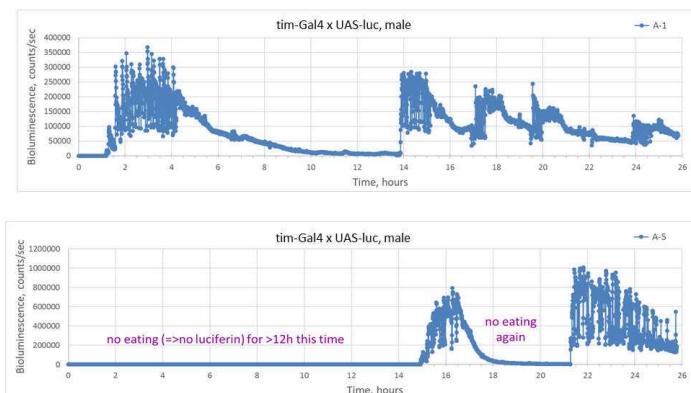


Fig. 9. C) Representative flies that have started to eat more than 1 and more than 14 hours after being placed into the plate (5% sucrose, 1% agar, 3 mM luciferin). Only the first 26 hours of recordings are shown.



Compared to 5% w/v sucrose, flies do not eat plain agar food and prefer not to eat low sucrose (0.5% w/v) food until really hungry (>12 hours).

Experiments with plain agar food show that bioluminescence monitoring can also be used to determine when flies become incapacitated or dead.

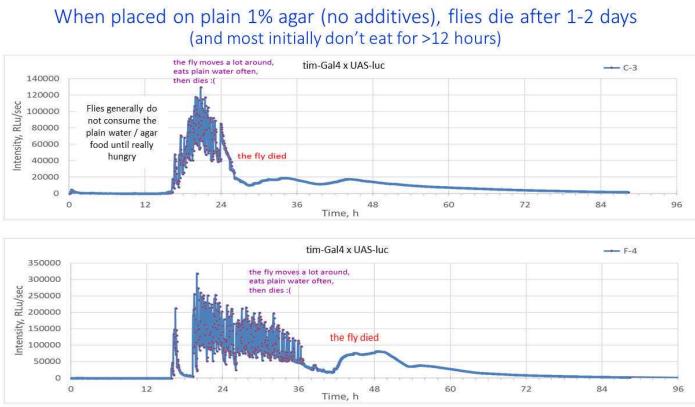


Fig. 9. D) Feeding behavior when placed on plain 1% agar (3 mM luciferin).

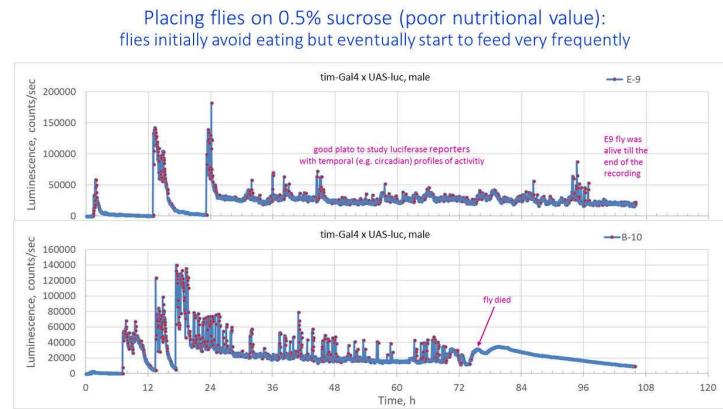


Fig. 9. E) Feeding behavior when placed on 0.5% sucrose, 1% agar (3 mM luciferin).

Low sugar food (0.5% sucrose) forces flies to eat very frequently. This often leads to flat luminescence curves but the extent of the effect depends on fly genotypes: in many cases flies will still show intervals of non-feeding.

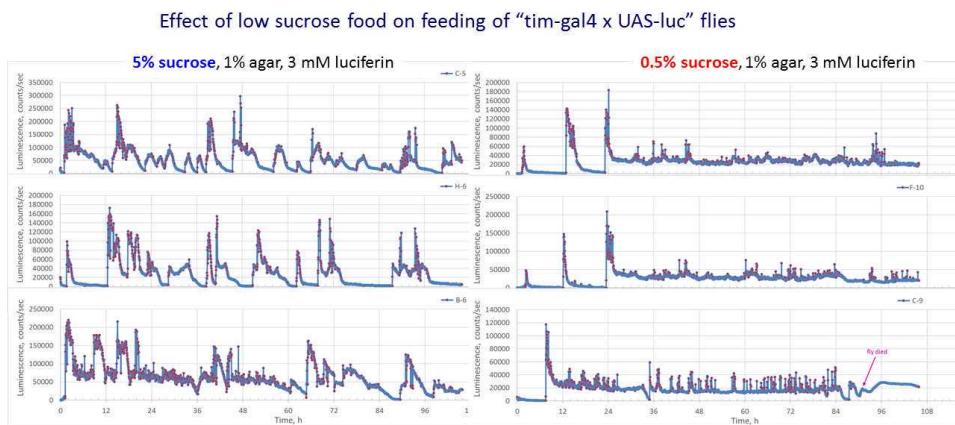


Fig. 10. A) Effect of low (0.5%) vs regular (5%) sucrose on feeding behavior of tim-Gal4xUAS-luc flies. 3 representative flies per condition are shown.

Effect of low sucrose food on feeding of "MEF2-gal4 x UAS-luc" flies

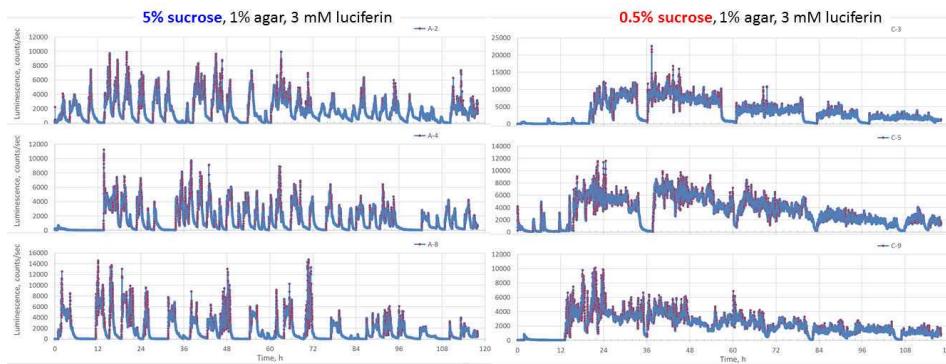


Fig. 10. B) Effect of low (0.5%) vs regular (5%) sucrose on feeding behavior of MEF2-Gal4xUAS-luc flies. 3 representative flies per condition are shown.



Individual flies can have different feeding patterns. Some individuals may show a very nice circadian feeding behavior with large fasting intervals.

Illustrative signal curves for individual flies

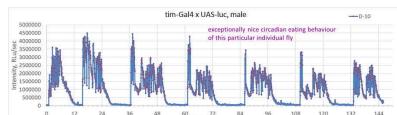


Fig. 11. A) Two exemplary flies.

Illustrative signal curves for individual flies

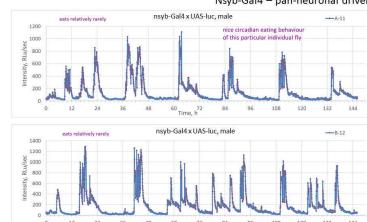


Fig. 11. B) Two exemplary flies.



Note 4. The classical use of luciferase as a transcriptional reporter in live flies and problems associated with it.

This insert illustrates and discusses typical results when recordings are performed according to the standard circadian reporter protocol. In this case multiple plates are measured by an automatic loading and transfer between two vertical plate stacker columns with each plate periodically being loaded into the platereader detection chamber.

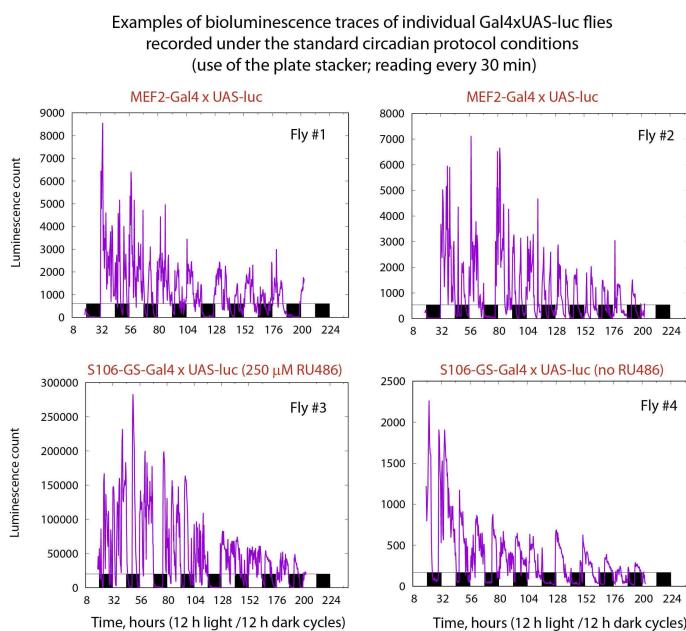
In this circadian reporter protocol, several features induce flies to eat more often which is a premise for using this method to record circadian gene expression reporters in live flies. Three main factors seem to induce flies to eat frequently:

1. Periodical loading the plate into the vertical stacker outside the instrument seems to disturb flies and stimulate them to eat more often.
 2. In some protocol implementations flies are placed under small plastic domes inside wells ([Stanewsky, 2006](#); [Yu & Hardin, 2007](#)) which induces flies to eat more frequently (likely, again by disturbing them).
 3. Likely, the formation of a water condensate on the film (if occurs) also can disturb flies and stimulate them to eat more often.

The efficiency of all these factors can vary depending on genotypes and not all fly individuals may be affected to the same extent.

More often than not the feeding rhythms still seem to persist through this procedure. Quite commonly, under these conditions typical separate feeding events in individual flies merge into a continuous bioluminescence curve which has two 12h peaks and was reported in early papers on the method (see Fig. 6A in Plautz et al (1997) and Fig. 2A in Brandes et al (1996)).

Such 12-hour patterns are often observed for Gal4xUAS-luc flies when recorded using the stacker method. See the Figure 12 below.



Many individual flies show the 12h rhythms in bioluminescence due to feeding under these recording conditions => not particularly suitable as transcriptional reporters which should change luminescence as a result of transcriptional differences

Fig. 12. Individual flies often still retain discrete feeding-related bioluminescence peaks under the standard circadian recording protocol (the stacker method) and often display 12 hour rhythms of bioluminescence.

When switching from a continuous recording to the stacker method (Fig. 13A), the bioluminescence profile (for constant luciferase levels in Gal4 lines) tends to switch from separate bioluminescence peaks into a continuous curve - from non-oscillating (*an ideal expected result*) to having high amplitude 12 hour rhythms (*a commonly observed artifact*).

tim-Gal4xUAS-luc: two types of bioluminescence curves during discontinuous recordings using the stacker

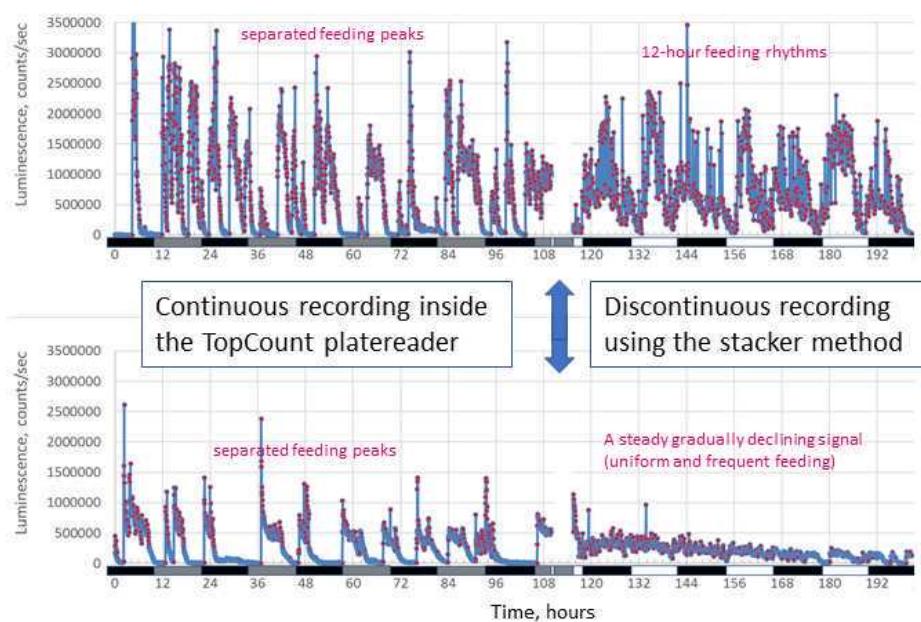


Fig. 13. A) Bioluminescence profiles of two individual tim-gal4xUAS-luc flies illustrating two types of curves that can be often observed when recording using the stacker method. The curves can depend on experimental conditions and on the individual flies.
(tim-Gal4xUAS-luc flies maintain a steady level of luciferase protein throughout the day)

It seems that some fly genotypes are more prone to maintain constant frequent feeding than others under these conditions. For example, the circadian BG-luc reporter flies indeed seems to display the expected intrinsic circadian expression rhythms when switched to a stacker-based recording protocol (Fig. 13B). However, as can be noticed, additional 12h-rhythm is present where the contribution of fly vertical movement seems to be primary source of the additional peaks (Fig. 13B).

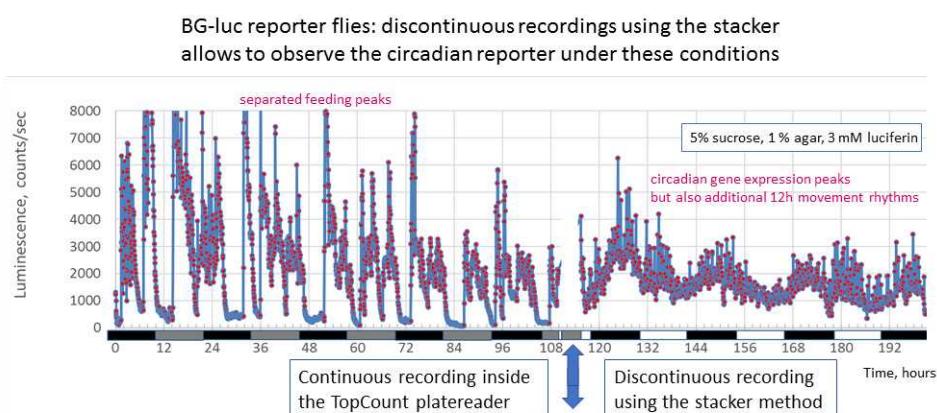


Fig. 13. B) BG-luc flies can display circadian rhythms of luciferase gene expression when recorded using the stacker method. A representative individual fly is shown.
As can be seen, at low sampling rates it would have been hard to discern the gene expression and the vertical movement which could lead to additional detected peaks.

Overall, this shows that the common implementation of this reporter system is poorly suitable to track gene expression (especially, circadian) in live flies: very rigorous controls and analyzing individual flies are required to discriminate feeding/movement and gene expression when using the original method or interpreting results obtained by it.

Some potential ways to solve this problem are discussed in the Note 1 (The Guidelines / Historical background section).