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Comparison Between Bioluminescence Imaging Technique and CFU Count for the Study of Oropharyngeal Candidiasis in Mice

Elena Gabrielli,[1] Elena Roselletti,[1] Eugenio Luciano,[1] Samuele Sabbatini,[1] Paolo Mosci,[2] Eva Pericolini[1]*

[1]Microbiology Section, Department of Experimental Medicine, University of Perugia, Perugia, Italy

[2]Internal Medicine, Department of Pathology, Diagnostic and Veterinary Clinic, University of Perugia, Perugia, Italy

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Correspondence to: Eva Pericolini, PhD, Microbiology Section, Department of Experimental Medicine, University of Perugia, Polo Unico Sant' Andrea delle Fratte, 06132, Perugia, Italy.
E-mail: pericolinie@hotmail.it

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Abstract

We recently described a bioluminescence in vivo imaging technique, representing a powerful tool to test the real-time progression of oropharyngeal candidiasis, hence potentially useful to evaluate the efficacy of antifungal therapies. In this study, the in vivo imaging technique was compared with CFU measurement of target organs (tongue, esophagus and stomach) for monitoring and quantifying oropharyngeal candidiasis. We have correlated these two analytical methods at different times post-infection using engineered, luminescent *Candida albicans* in mice rendered susceptible to oral candidiasis by cortisone-acetate. Scatter plots, Pearson correlation and Student's *t* test were used to compare the methods. We observed that the bioluminescence in vivo imaging technique was more reliable than CFU counts in detecting early infection of, and its extent in, the oral cavity of the mouse. This was also evident following the introduction of a variable such as treatment with fluconazole. The results described in this study could validate the bioluminescence in vivo imaging technique as a method to monitor and quantify oropharyngeal candidiasis and to assess early discovery of active compounds in vivo. © 2015 International Society for Advancement of Cytometry

Key terms

OPC; bioluminescence; correlation; CFU

A few specialized *Candida* species reside as harmless commensal organisms within the normal oral, gastrointestinal and vaginal microbiota in approximately half of the world's population (1). Although normally these fungi cause no pathology, under suitable predisposing conditions, including long-term antibiotic treatment and compromised local immune-barrier defenses, these fungi may become pathogenic, giving rise to severe morbidity in millions of individuals worldwide (1,2). In particular, *Candida albicans* (*C. albicans*) is present in the oral cavity of up to 75% of the population. In healthy individuals this colonization generally remains benign. However, mildly immunocompromised individuals can frequently suffer from recalcitrant infection of the oral cavity. This infection is termed oral candidiasis and can spread to the pharynx and/or the esophagus (oropharyngeal candidiasis, OPC) of persons with dysfunction of the adaptive immune system, particularly in HIV-infected subjects (3).

We recently described the dissemination of *C. albicans* from the oral cavity to the gastrointestinal tract (4) by in vivo imaging and CFU counts of target organs (tongue, esophagus, stomach, and intestine) (5). Data suggested that the bioluminescence in vivo imaging technique was more sensitive than the classic CFU method because of the prominent hyphal growth of *C. albicans* in vivo resulting in low plating efficiency. Hyphal mats may be counted as single colony, causing a severe underestimation of the fungal load (6). In particular, the oral mucosa includes the tongue,

gingival tissues, soft palate and pharynx, hence CFU determination of the tongue only might not be completely accurate for quantifying the real fungal burden of the whole oral cavity (4).

To validate the in vivo imaging technique as alternative method to monitor OPC, we have here addressed a correlation study by a direct comparison of the two analytical methods, i.e., bioluminescence in vivo imaging and CFU at different time points post-infection. This was done by injecting engineered, luminescent *C. albicans* in mice rendered susceptible to oral candidiasis by treatment with cortisone-acetate. Moreover, bioluminescence and CFU were also compared in excised target organs (esophagus and stomach). Scatter plots, Pearson correlation and Student's *t* test were used to match the methods. We also considered fluconazole (FLZ) treatment to further validate the method of in vivo imaging.

The results of our study show that the bioluminescence in vivo imaging technique is a reliable and sensitive method for early detection and assessment of the extension and duration of OPC. In particular, this method positively compares with the usual methods of assessing OPC by tongue CFU counts and histology, even following antimycotic treatment.

Materials and Methods

Candida albicans Strain and Culture

Candida albicans CA1398 carrying the *ACT1p-gLUC59* fusion (gLUC59) was used (7). The *gLUC59* luciferase reporter has previously been described (7). Briefly, the luciferase reporter gene was constructed from the coding sequence of the *C. albicans PGA59* gene, encoding a GPI-linked cell wall protein, and the *Gaussia princeps* luciferase gene. *C. albicans* gLUC59 was cultured in YPD (yeast peptone dextrose) broth as described by Solis et al. (8).

Ethics Statement

The procedures involving the animals and their care were conducted in conformity with the national and international laws and policies. All animal experiments were performed in agreement with the EU Directive 2010/63, the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, and the National Law 116/92. The protocol was approved by Perugia University Ethics Committee for animal care and use (Comitato Universitario di Bioetica, permit number 149/2009-B). All the animals were housed in the animal facility of the University of Perugia (Authorization number 34/2003A). Mice were acclimatized for a week before starting the experiments. Five to eight mice were housed in each cage and were provided with food and water ad libitum. All efforts were made to minimize suffering during experiments. In particular, to monitor the clinical course of the infection each day, clinical parameters used to assess the general health status are employed by animal care taker such as: body weight and temperature, behavior, fur coat and posture, loss of appetite and dehydration. Humane endpoint was defined considering: prolonged loss of appetite (>48 h), signs of dehydration, inability to ambulation and consequent difficulty to reach food and water, difficulty in maintaining an up-right position. Mice were euthanized when positive to three of these clinical signs. On this basis the experiment was terminated at day +8 post-infection, in order to prevent unnecessary suffering. Mice were euthanized through intraperitoneally injection of 200 μl Ketamin/Xylazin until the animal stops breathing. The visual inspection post-mortem of the body surface and orifices showed no abnormalities except those due to infection (see below) such as the presence of plaques on the oral cavity.

Mouse Model of OPC

Female, 6- to 8-weeks old, inbred C57BL/6 mice were obtained by Harlan Nossan Laboratories, Milan, Italy. Mice were treated subcutaneously (s.c.) with 225 mg kg^{-1} cortisone acetate (Sigma–Aldrich) in a total volume of 0.2 mL and all were infected by placing a calcium alginate swab (Fisher Scientific, Illkirch Cedex, France) soaked with 1×10^6/ml *C. albicans* blastopore suspension sublingually for 75 min as previously described (8). As a control, selected mice were treated by placing a calcium alginate swab soaked with saline (mock-infected mice). Infections were conducted under anesthesia with a s.c. injection of a mixture of Tiletamine/Zolazepam-Xylazine (50–5 mg kg^{-1}). The oral cavity was swabbed immediately before the infection and streaked on YPD agar plus chloramphenicol (50 μg ml^{-1}) (both from Sigma–Aldrich) to verify the absence of *Candida spp.* In selected experiments, anesthetized infected mice were treated sublingually with fluconazole (FLZ) (40 μg/10 μl mouse) at day +1 and +2 post-infection.

Real-time Monitoring of OPC

At selected days, starting on day +3 after challenge, 10 μl (0.5 mg ml^{-1} in 1:10 methanol:H_2O) of coelenterazine (Synchem, OHM) was added sublingually in anesthetized mice. Mice were then imaged in the IVIS-200TM Imaging system (Xenogen Inc.) under s.c. anesthesia. The total photon emission from oral areas within the images (Region Of Interest, ROI) of each mouse was quantified with Living ImageR software package as previously described (4,5). An irrelevant background of luminescence was observed in uninfected mice treated with coelenterazine (mean ± SEM of seven different mice: 90965,43 ± 13509,55). An *ex vivo* analysis of pharynx, esophagus and stomach from mice with OPC was performed. After 3, 6, and 8 days post-infection mice were euthanized, the gastric tracts (pharynx, esophagus and stomach) were excised and 10 μl (0.5 mg ml^{-1}) of coelenterazine (Synchem) was injected through the pharynx into the esophagus lumen, to visualize the fungal burden using the IVIS-200TM Imaging system as described above. After 3 days post-infection an *ex vivo* analysis of tongue of mice with OPC was performed. Briefly, tongue was excised from euthanized mice and soaked with 10 μl (0.5 mg ml^{-1}) of coelenterazine (Synchem) to visualize the fungal burden as above described.

In Vitro Quantification of Bioluminescence

C. albicans cells, grown in liquid culture of YPD or Sabouraud (SAB) for 24 h at 30°C, were collected by centrifugation, washed twice in luciferase assay buffer (LA) as previously

described (7). Coelenterazine in LA buffer was added to a final concentration of 1.25 μM, and luciferase activity was recorded using a luminometer. To assay *C. albicans* cells in microtiter plate format, a dilution series were prepared for each culture, and 100 μl of each dilution was deposited in black 96-well microtiter plates with transparent bottom and mixed with 100 μl of 2 μM coelenterazine in LA buffer.

CFU Assay

The fungal burden of the tongue, esophagus and stomach 3, 6, and 8 days post-infection was evaluated by plating serial dilutions of organ homogenates onto YPD agar plus chloramphenicol (50 µg ml^{-1}) (both from Sigma–Aldrich) and counting colony forming units (CFU). Briefly, after aseptic removal, all organs were weighed, homogenized in 3 mL of sterile saline and 100 μl of undiluted samples or 100 μl of 10-fold serial dilutions were plated and incubated at 37°C for 48 h. The fungal load was expressed as the number of CFU per gram of organ. About 1 or 25–250 CFU for plate were considered as Detection or Quantification Limits, respectively (see: http://www.microbiol.org/wp-content/uploads/2010/07/Sutton.jvt_.2011.17_3.pdf). In selected experiments, to assess bioluminescence activity, *C. albicans* colonies from organ homogenates growing onto YPD agar plus chloramphenicol were replicated onto 3 M filter papers. One milliliter of LA buffer containing 1.25 μM coelenterazine was then deposited on the filter article. The filter was imaged with the IVIS-200TM (data not shown). Moreover, in selected experiments, tongue homogenates were tested for bioluminescence prior to plating by adding 20 µl of coelenterazine (0.5 mg ml^{-1}) in an eppendorf containing 500 µl of tongue homogenates. Concomitantly, 100 µl of undiluted tongue homogenates were plated in YPD plus chloramphenicol and incubated at 37°C for 48 h. After incubation, growth of bioluminescent colonies was visualized by bioluminescence imaging using an IVIS-200TM. To start the reaction, 3 ml of LA buffer containing 30 μl of coelenterazine (0.5 mg ml^{-1}) were added. Moreover, 200 µl of the same undiluted tongue homogenates were seeded in black 96-well microtiter plates with transparent bottom and 2 μl of coelenterazine (0.5 mg ml^{-1}) were added then luciferase activity was recorded using a luminometer.

Histological Analysis

Six days post-infection the animals were sacrificed to analyze gross and histopathologic lesions. The posterior portions of the tongue, pharynx, and gingival tissues were excised, fixed immediately in 10% formalin and embedded in paraffin. The posterior portions of the tongue and pharynx were sectioned longitudinally and gingival tissues transversally to verify the extension of the lesions. The 3- to 5-µm thick sections were stained using the periodic acid-Schiff (PAS) procedure to visualize fungi, and examined by light microscopy (Leica DM2500). The scale bars are in μm.

DNA Extraction of *C. albicans* gLUC59 from Infected Tongues and Quantitative PCR Analysis

Genomic DNA was extracted from the homogenized tongues of mice at day +3, +6, and +8 post-infection with *C. albicans* gLUC59 or mock-infected using Trizol reagent (Invitrogen). DNA concentration was determined using a spectrophotometer (Eppendorf Bio Photometer plus). Mouse GAPDH gene was detected using Forward primer (5′–3′): GCCTTCCGTGTTCCTACCC and Reverse primer (5′–3′): CAGTGGGCCCTCAGATGC and specific *C. albicans* ACT1 gene using Forward primer (5′–3′): GACAATTTCTCTTTCAGCACTAGTAGTGA and Reverse primer (5′–3′): GCTGGTAGAGACTTGACCAACCA. Real-time PCR (quantitative PCR) was performed in 96-well PCR plates using the SYBR green (all from BioRad). For real-time PCR reaction 200 ng of DNA was used. All samples were measured in six replicates and DNA quantities reported as $1/\Delta C_T$ values ($1/(C_T$ from *C. albicans* ACT1 gene—C_T from mouse GAPDH)). Amplification conditions were the same used for ACT1 and GAPDH genes: 3 min at 95°C, 40 cycles of 10 s at 95°C and 30 s at 55°C. The experiments were performed using the Eppendorf Mastercycler.

Statistical Analysis

The course of OPC in fifteen mice was monitored by bioluminescence imaging in each single mice from day +3 to day +8. In parallel, at each time point, fifteen mice were euthanized, tongue was excised and fungal burden determined by CFU. To verify the fungal burden in excised organs ten mice for each time point were euthanized, esophagus and stomach were excised, analyzed by bioluminescence imaging and CFU enumeration. CFU count was reported as CFU/g tissue; data of esophagus and stomach were summed up. To compare both methods during FLZ treatment, data are from ten mice. Scatter plots, Regression coefficient (R^2), Pearson correlation (r) and two-tailed Student's t test were used to compare total photon flux emission and CFU count in target organs. Data were presented as means ± SEM and SD from three independent experiments. To estimate the correlation between the two analytical methods, r from linear regression models was used. Values of $P < 0.05$ were considered significant according to two-tailed Student's t test using Microsoft Excel software.

Results

Previous studies quantifying fungal load in the oral cavity used colony forming unit (CFU) counts of tongue taken as representative of the whole oral cavity (8,9). We recently used a bioluminescent in vivo imaging technique to monitor oropharyngeal candidiasis (OPC) in mice (4,5). In the present study, we compared CFU and bioluminescence in experiments carried out in mice infected with *C. albicans* and monitored with both methods at 3, 6, and 8 days post-infection.

As shown in Table 1, the CFU method failed to detect fungal burden in the tongue of six and seven out of fifteen mice after 3 and 6 days post-infection, respectively. The presence of fungal cells in the tongue of all mice was detected by the CFU method only on day 8 after challenge. In contrast, the bioluminescence technique revealed fungal infection in the oral cavity of all mice at each time point tested (Table 1). In addition, on day +6 post-infection, tongue CFU counts in positive animals were roughly the same as those on day 3

Table 1. Fungal load of oral cavity measured by two methods: oral bioluminescence and CFU from tongue*

DAY +3		DAY +6		DAY +8	
TOTAL PHOTON FLUX ($\times 10^6$)	CFU/G TISSUE	TOTAL PHOTON FLUX ($\times 10^6$)	CFU/G TISSUE	TOTAL PHOTON FLUX ($\times 10^6$)	CFU/G TISSUE
8.196	33.0	7.712	1787.0	44.920	786.0
2.519	231.0	4.469	1985.0	20.450	1252.0
4.834	99.0	23.430	3069.0	52.030	1165.0
2.343	8476.0	19.980	6550.0	32.230	1096.0
55.290	9333.0	32.850	4477.0	142.800	1356.0
2.055	2377.0	4.354	3303.0	6.442	1043.0
1.057	2377.0	1.541	63.0	14.070	243.0
2.053	976.0	1.180	42.0	91.210	243.0
1.282	698.0	4.141	0	124.300	1702.0
2.654	0	0.909	0	44.470	1255.0
2.962	0	8.669	0	3.528	8500.0
3.500	0	8.807	0	5.400	7250.0
8.744	0	91.400	0	56.600	5617.0
6.521	0	47.120	0	80.220	4255.0
34.890	0	98.840	0	31.140	18729.0
10.660[a]	**1640.0**	**23.420**	**1418.4**	**49.990**	**3632.8**
± 3.873[b]	± 764.4[b]	± 7.992[b]	± 517.5[b]	±10.698[b]	±1228.7[b]
±14.996[c]	±2959.8[c]	±30.948[c]	± 2003.9[c]	±41.425[c]	±4757.7[c]

*Fungal burden from fifteen different mice quantified by both methods.
[a]Means.
[b]± SEM.
[c]±SD.

post-infection but bioluminescence doubled over the same time interval. This enhanced sensitivity of the bioluminescence method was also seen on day +8 (Table 1). Despite the differences in positivity of infection measured by the CFU counts in some mice and the high variability of both CFU and photon flux data, the Pearson correlation test showed a substantial correlation between the two methods at 3 days post-infection ($r = 0.6968$; $P = 0.0186$). However, no or a negative correlation was evidenced at day +6 and +8 post-infection (Supporting Information Fig. 1). The overall correlation between the two methods, taking into account all determinations at each time point, was weakly positive, as assessed by the Pearson correlation coefficient ($r = 0.044$; $P = 0.000003665$).

Because the data above suggested an imperfect match between bioluminescence determination and CFU counts, a direct comparison of the two methods was sought for. To this aim, we first examined whether fungal growth in vitro, both in a nutrient relatively rich (YPD) and in a poor (SAB) growth medium, could differently affect the determination of fungal load by the two methods. In particular, the bioluminescence emission could be affected by compounds of the medium influencing promoter activity of *C. albicans* gLUC59. As shown in Figure 1A, there was a very good correlation between cell number and bioluminescence in both culture media. Then, we compared face to face tongue homogenates from two different mice on days +3, +6, and +8 post-infection by bioluminescence and CFU enumeration (an *ex vivo* bioluminescence analysis of a representative isolated, infected tongue at day +3 post-infection is shown in Fig. 1B). As shown in Figure 1C, not all tongue homogenates resulted positive to bioluminescence and to CFU counts (days +3 and +6 post-infection) and, in this case, the bioluminescence emission and CFU from tongue homogenates were under detectable levels (but fungal DNA was detected in those homogenates, see below). However, with increasing fungal load on tongue, both the homogenates and the colonies resulting from plating the homogenates were bioluminescent (day +8 post-infection). Overall, bioluminescence and CFU counts showed good correlation when the same tissue samples were examined (i.e., excised tongue) (Fig. 1C).

Luciferase activity of the same tongue homogenates, expressed as relative luminescence units (RLU) and reported in panel D of Figure 1, revealed the same trend of bioluminescence emission reported in panel C of Figure 1. To further strengthen these results the same tongue homogenates were analyzed by qPCR assay detecting abundance of ACT1 gene from *C. albicans* and normalized against mouse GAPDH gene. The data are reported as $1/\Delta C_T$ a parameter representative of the relative DNA amount (see Materials and Methods). The results reported in panel E of Figure 1 show the expected presence of fungal DNA in all tongue homogenates at each time point tested even when the tongue is poorly infected and negative with both CFU and bioluminescence assays. Incidentally, and as expected, the most sensitive method derives from DNA quantification; however, threshold values have not been shown here. In agreement with previous investigations from other research groups (10,11) mock-infected mice resulted negative for fungal DNA (not shown).

The differences noticed above between detection and quantitation of fungal infection by the two methods (tongue

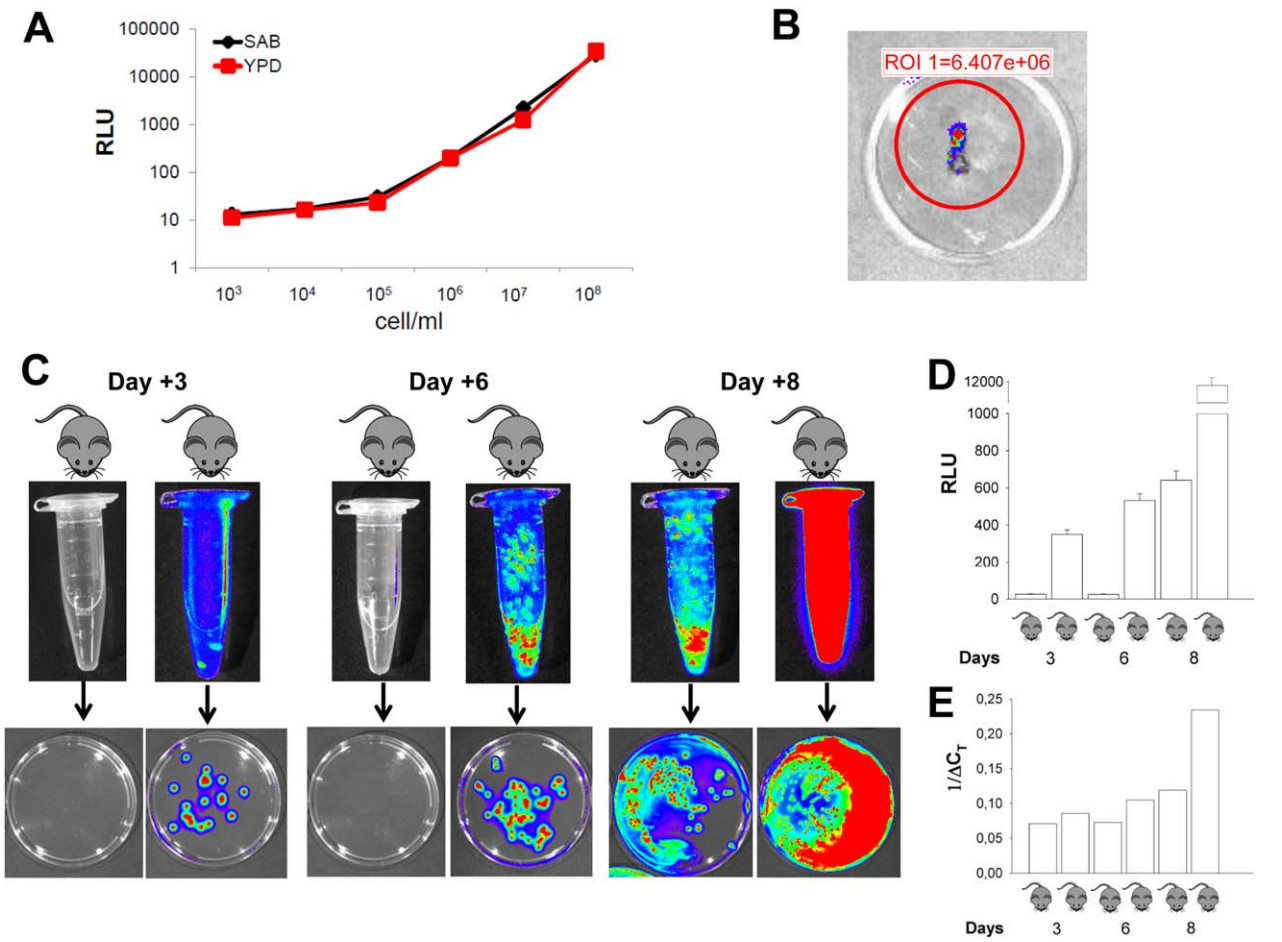

Figure 1. *Ex vivo* bioluminescence analysis of tongue, in vitro quantification of bioluminescence and quantification of fungal DNA. Increasing number of *C. albicans* gLUC59 cells grown in YPD or SAB medium at 30 °C were incubated in LA buffer. Luciferase activity was measured using intact cells with coelenterazine as substrate, Relative Luminescence Units (RLU) are shown (**A**). An *ex vivo* bioluminescence analysis of a representative isolated, infected tongue at day +3 post-infection is shown (**B**). At 3, 6, and 8 days post-infection tongue homogenates from two different mice were analyzed for bioluminescence prior and after plating (**C**). Relative Luminescence Units (RLU) of tongue homogenates from two different mice at day +3, +6, and +8 post-infection were also analyzed using luminometer (**D**). For the quantification of each PCR product, the threshold cycle (C_T) was used. The ΔC_T value was calculated from the difference in the C_T of *C. albicans* ACT1 gene and that of mouse GAPDH gene. Data are presented as $1/\Delta C_T$ value (six measurements from two different mice at day +3, +6, and +8 post-infection (**E**).

CFU and bioluminescence of the whole oral cavity) suggested that they could be due to the presence of foci of infection in different sites of oral cavity, detected by the bioluminescence in vivo and obviously not detected by tongue CFU examination. Thus, we performed an histological analysis of whole oral cavity at day +6 post-infection. This analysis revealed the presence of *C. albicans* foci of infection in the posterior portion of the tongue, pharynx and gingival tissues (Fig. 2). *C. albicans* blastospores and invasive hyphae were present in the posterior portion of the tongue, pharynx and gingival tissues of infected mice at the level of keratinized superficial epithelium. This was accompanied by infiltration of inflammatory cells (Fig. 2). To further assess the efficacy of both methods in the evaluation of OPC progression, we also compared the sensitivity of both methods to measure fungal burden in the excised target organs, esophagus and stomach, at day +3, +6, and +8 post-infection.

Of the ten excised target organs (esophagus and stomach) of each time point tested, the CFU method was able to detect the fungal load in nine organs after 3 and 6 days post-infection and in all ten excised organs after 8 days (Table 2). Fungal load from all organs were successfully measured by the *ex vivo* bioluminescence technique at all time points tested (Table 2). Both methods revealed a time-dependent increase of fungal burden, particularly evident between day +6 and day +8 (Table 2). In line with this evidence, our data showed that there was a statistically significant association between CFU and bioluminescence after 3 days post-infection ($r = 0.5765$; $P = 0.0000022$) (Fig. 3). At late time points post infection (day +6 and +8), the values of Pearson correlation showed a trend for lower values ($r = 0.2661$; $P = 0.0000017$ day +6 post-infection and $r = 0.3385$; $P = 0.013$ day +8 post-infection), with, however, still good levels of correlation (Fig. 3).

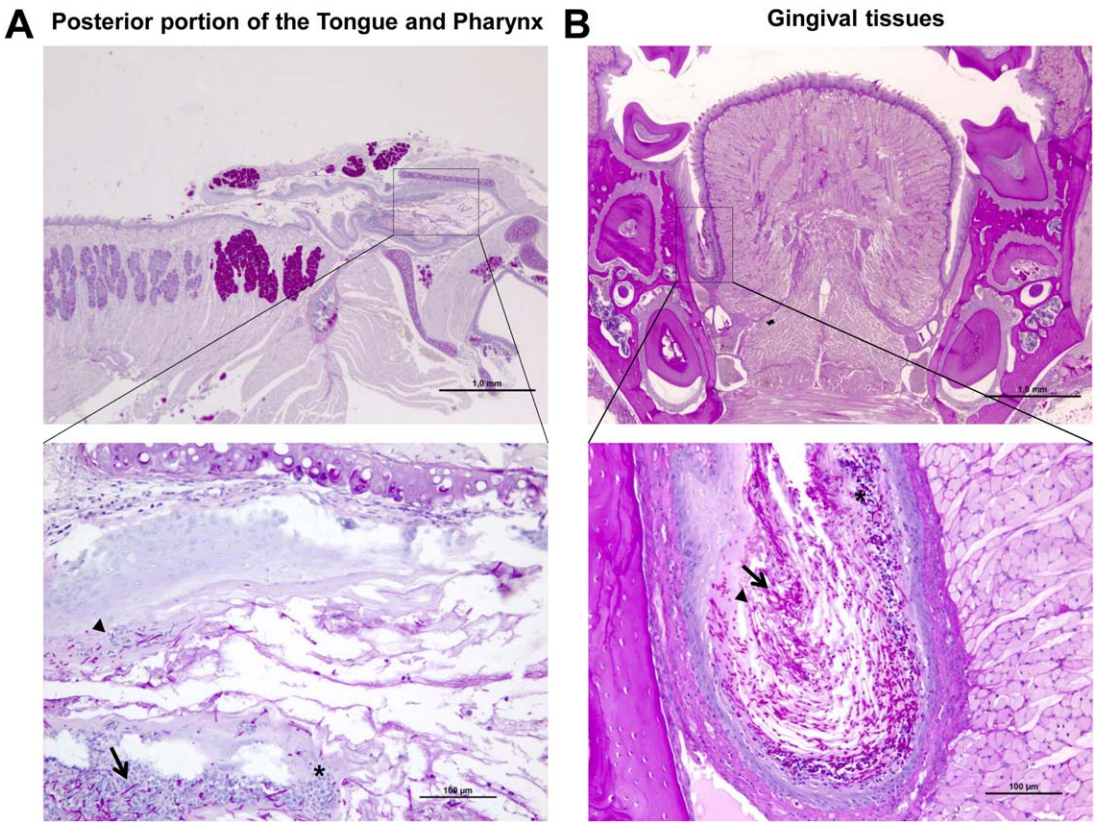

Figure 2. Histopathology of posterior portion of the tongue, pharynx, and gingival tissues. Tissue sections from infected mice are shown (day +6 post-infection). Posterior portion of the tongue and pharynx (**A**, left panels, scale bar 1.0 mm and enlargement view 100 μm) and gingival tissues sections (**B**, right panels, scale bar 1.0 mm and enlargement view 100 μm) show infiltration of inflammatory cells (asterisk) and some invasive fungal hyphae (arrow) and blastospores (head arrow) in the keratinized superficial epithelium.

The overall correlation across the different time points for the *ex vivo* target organs revealed, again, good level of positive Pearson correlation ($r = 0.6297$; $P = 0.002083$).

Given that the best correlation of fungal load between the two analytical methods was noticed at day +3 post-infection, we further analyzed if this relationship did also occur during

Table 2. Fungal load of esophagus and stomach measured by two methods: bioluminescence and CFU from excised organs*

DAY +3		DAY +6		DAY +8	
TOTAL PHOTON FLUX ($\times 10^6$)	CFU/G TISSUE	TOTAL PHOTON FLUX ($\times 10^6$)	CFU/G TISSUE	TOTAL PHOTON FLUX ($\times 10^6$)	CFU/G TISSUE
0.081	19.0	0.217	12.0	1.078	1852.0
0.113	186.0	0.121	12.0	0.216	1926.0
0.082	19.0	0.166	60.0	0.293	1778.0
0.137	262.0	0.134	205.0	0.232	17534.0
0.132	18.0	0.265	256.0	0.302	20834.0
0.092	20.0	0.188	840.0	0.879	20450.0
0.109	18.0	0.205	916.0	0.732	17336.0
0.079	12.0	0.248	117.0	0.828	26421.0
0.106	12.0	0.129	94.0	1.071	22511.0
0.039	0	0.130	0	3.286	22210.0
0.097[a]	**56.6**	**0.180**	**251.2**	**0.892**	**15285.2**
± 0.0087[b]	± 27.0[b]	±0.015[b]	±102.3[b]	± 0.27[b]	±2885.1[b]
± 0.027[c]	± 85.5[c]	± 0.049[c]	± 323.6[c]	±0.86[c]	±9122.8[c]

*Fungal burden from ten different excised organs quantified by both methods.
[a]Means.
[b]± SEM.
[c]± SD.

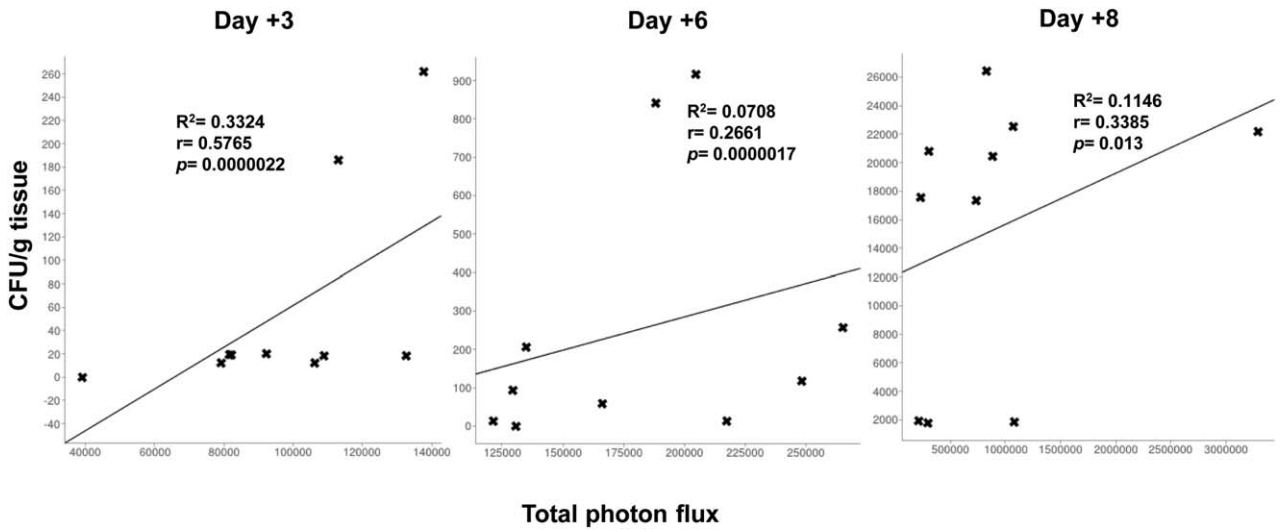

Figure 3. Scatter plot of fungal load in the esophagus and stomach in ten study organs for each time point tested (day +3, +6, and +8 post-infection) measured by total photon flux and CFU. Linear regression lines are shown. Regression coefficient (R^2), Pearson correlation (r) and statistical significance are indicated in each panels. $P < 0.05$ according to Student's t test.

FLZ treatment, which is the gold standard in the treatment of oral candidiasis (6,12). To this end infected mice were treated sublingually with FLZ at day +1 and +2 post-infection, then fungal load in the oral cavity was measured by both techniques. At day +3 post-infection, the expected reduction in bioluminescence was corroborated by plating tongue homogenates, which confirmed a correspondent reduction in fungal burden by the CFU measurement (Fig. 4A and Table 3). At the above time point the fungal load was relatively low (average 54.3 CFU/g tissue), yet bioluminescence was clearly detectable in the oral cavity of live mice, representing the lowest number of yeasts that we were able to detect in vivo (Fig. 4 and Table 3).

The Pearson correlation coefficient ($r = 0.6084$; $P = 0.03591$) clearly demonstrates that both methods are substantially correlated during antifungal therapy (Fig. 4B). Nonetheless, we observed a statistically significant reduction in the fungal load of oral cavity after 3 days of FLZ treatment as compared to untreated controls only in mice monitored by the bioluminescence method ($P = 0.0483$) (Fig. 4C). These

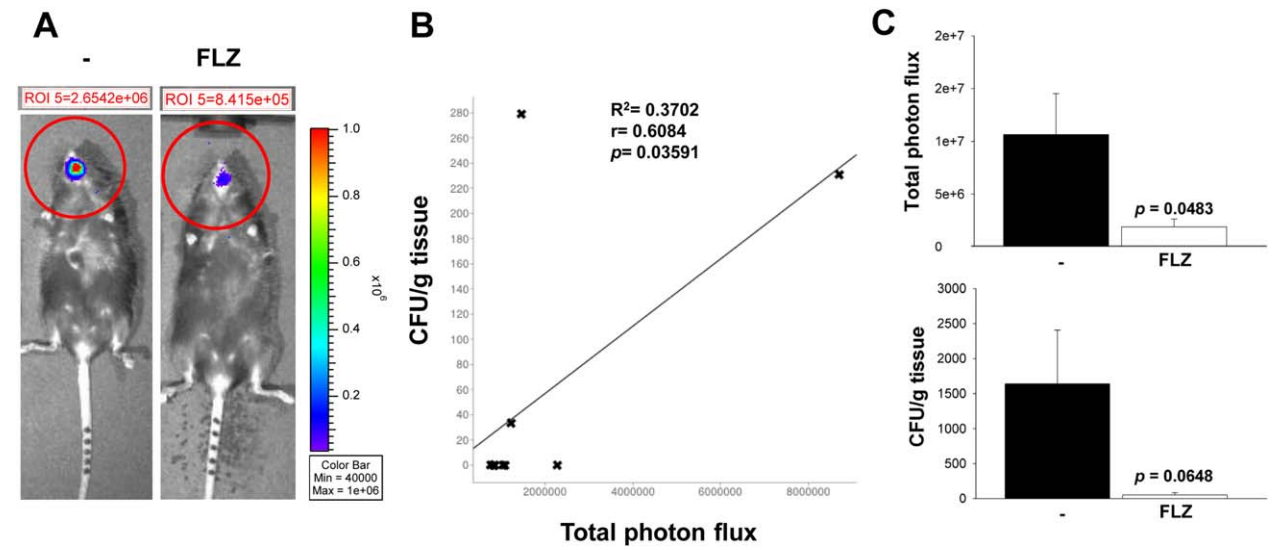

Figure 4. Evaluation of oral candidiasis in mice at day +3 post-infection during FLZ treatment measured by total photon flux and CFU from tongue. Representative image of bioluminescence of murine oral candidiasis at day +3 post-infection, in the presence or absence of FLZ treatment, is shown (**A**). Linear regression line is shown. Regression coefficient (R^2), Pearson correlation (r) and statistical significance are indicated. $P < 0.05$ according to Student's t test (**B**). Fungal burden in the oral cavity of mice treated or untreated with FLZ, at day +3 post-infection determined by total photon flux (upper panel) or by tongue CFU (lower panel) are shown (**C**). Means ± SEM are shown. P values were determined according to Student's t test.

Table 3. Fungal load of oral cavity measured by two methods: oral bioluminescence and CFU from tongue at day +3 post-infection during FLZ treatment*

TOTAL PHOTON FLUX ($\times 10^6$)	CFU/G TISSUE
1.447	279.0
2.271	0
0.818	0
0.751	0
0.841	0
0.815	0
8.705	231.0
1.025	0
1.074	0
1.221	33.0
1.897[a]	**54.3**
± 0.730[b]	± 32.0[b]
± 2.309[c]	± 101.3[c]

*Fungal burden from ten different mice quantified by both methods.
[a]Means.
[b]± SEM.
[c]±SD.

results suggest that bioluminescent *C. albicans* gLUC59 could be applied to rapid in vivo testing of new antifungal compounds in the oral candidiasis.

Altogether, the whole set of our experimental data suggests that bioluminescence provides a superior analysis of the oral cavity infection at early stages, when low, inconsistent levels of tongue infection and difficulties in monitoring by the CFU method, ill-defined sites of the oral cavity (i.e., soft palate and gingival tissues), are present. The analysis of the entire oral cavity by real-time in vivo imaging technique allows rapid detection of infection in the oral cavity that is not located at the tongue. Bioluminescence thus appears as rapid method to assess the infection process in living animals and allows to monitor drug efficiency testing from small animal groups, as shown for disseminated candidiasis using firefly luciferase (13).

Discussion

CFU measurement of the tongue is generally used, in association with the histological analysis, to determine the degree of *C. albicans* infection of the oral cavity in different mouse model of OPC (8,9,14,15). We recently described for the first time a new approach for longitudinal monitoring of the dynamics of oral *C. albicans* infection by the use of an in vivo and *ex vivo* imaging technique exploiting stably transformed bioluminescent *C. albicans*. In principle, this approach represents a powerful tool to monitor real-time progression of infection from tongue to esophagus and stomach, identify the target site of *C. albicans* in specific organs and explore the spread of *C. albicans* from local to systemic compartment (4,5). These previous studies suggested that the CFU enumeration of the tongue may underestimate the real fungal load in the oral cavity during OPC and can miss determinants of infection spread at non-tongue sites of the oral cavity. In fact, CFU enumeration completely failed to detect the early spread of the *C. albicans* cells in other parts of the oral cavity such as gingival tissues, soft palate and pharynx (4).

The CFU enumeration of the tongue is routinely used to quantify murine OPC (8,9,14,15), but in our experimental conditions, it showed a large variability with numerous determinations below the threshold of detection and quantification. These values must be necessarily considered to make a realistic comparison with the method of the bioluminescence.

Despite all the known limitations of CFU methodology and the reports of the usefulness of bioluminescence methodology for monitoring fungal infection of the oral cavity, little has been done for a direct comparison of the efficiency and correlation of the two techniques. In this article, we have made a comparison of the above two methods during experimental oral candidiasis in mice. Overall, we noticed remarkable differences between the two methods to measure the fungal burden. In particular, the CFU method failed to measure early tongue colonization in a significant proportion of infected mice. In fact, and contrarily to what observed by other research groups that used different inoculum size of fungus and/or different mouse strain (8,9,16,17), the CFU method in our experimental setting failed to detect fungal burden in the tongue in a rather remarkable number (about half) of tested mice on days +3 and +6 post-infection, showing in general a lower sensitivity in determining the fungal load in the oral cavity. However, the two methods well matched in directly compared, excised target organs, when a sufficient density of fungal cells was present. This suggests that differences in the detection of infection in non-tongue tissues of the oral cavity are realistically the primary determinant of the greater sensitivity of the bioluminescence method in monitoring the infection of the whole oral cavity. In addition, the low CFU sampling efficiency of hyphal cells may contribute to the above difference. Because the oral mucosa includes the tongue, gingival tissues, soft palate and pharynx, CFU determination of tongue might not be completely accurate for expressing the real fungal load of the whole oral cavity. In addition, the physical interaction of *C. albicans* yeast cells with epithelial cells is a potent stimulator of germ-tube/hyphae formation (18,19), thereby intrinsically linking adhesion with hyphae formation in mucosal infection. *C. albicans* hyphae adhere more strongly to epithelial cells than yeast cells (1,20). Because *C. albicans* hyphae are well attached to the tissue, several hyphae often grew as single colony, causing an underestimation of the fungal load by the CFU method. Conversely, the bioluminescence in vivo imaging technique is able to assess hyphae, that are critical for the invasion process, in a more quantitative manner (21,22) thus allowing for a more sensitive evaluation of infection than standard CFU counting.

Although the use of a single and low inoculum size can be a limitation of the present study, it must be stressed here that we have used a steroid-treated model that is more representative of the human infection than other methods based on heavy inoculum sizes of fungal cells (3). Thus, our hypothesis of

bioluminescence in vivo imaging technique as a better method seems to be supported by the data presented in this study.

FLZ is often utilized as a control in drug screening experiments. We here provide for the first time a proof-of-concept of the feasibility of our bioluminescence-monitored mouse model to assess drug efficacy. With FLZ we have generally observed an effect on fungal growth similarly displayed by bioluminescence of oral cavity and tongue CFU. However, at early days post-infection, the bioluminescence method displayed a statistically significant difference between FLZ-treated and untreated mice, that was not shown in the mice monitored by the CFU counts. Therefore, bioluminescence imaging could be used for the initial rapid screening of compounds. Our results clearly demonstrated that in the presence of FLZ, the substantial correlation previously observed without treatment was maintained (see above), suggesting that the bioluminescence method can replace and still be more reliable of the tongue CFU in the monitoring of the effectiveness of antifungal therapy during OPC.

In summary, the data suggest that bioluminescence may be a reliable method to visualize and quantify the effect of antibiotic treatment. If necessary, the sensitivity of the technique can be increased by imaging excised organs *ex vivo*, offering a more rapid readout than traditional growth-based assays. We believe that the model presented here can be usefully exploited in future studies of mechanisms of invasion by *C. albicans*, as well as for early drug discovery as an easy and rapid way to identify active compounds in vivo.

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