**diskImageR**: quantification of resistance and tolerance to antimicrobial drugs using disk diffusion assays

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Microbial pathogens represent an increasing threat to human health. Although many infections can be successfully treated and cleared, drug resistance is a widespread problem. The existence of subpopulations of ‘tolerant’ cells (where a fraction of the population is able to grow above the population resistance level) may increase the rate of treatment failure; yet, existing methods to measure subpopulation effects are cumbersome. Here we describe diskImageR, a computational pipeline that analyses photographs of disk diffusion assays to determine the degree of drug susceptibility [the radius of inhibition, (RAD)], and two aspects of subpopulation growth [the fraction of growth (FoG) within the zone of inhibition, (ZOI), and the rate of change in growth from no drug to inhibitory drug concentrations, (SLOPE)]. diskImageR was used to examine the response of the human fungal pathogen *Candida albicans* to the antifungal drug fluconazole across different strain backgrounds and growth conditions. Disk diffusion assays performed under Clinical and Laboratory Standards Institute (CLSI) conditions led to more susceptibility and less tolerance than assays performed using rich medium conditions. We also used diskImageR to quantify the effects of three drugs in combination with fluconazole, finding that all three combinations affected tolerance, with the effect of one drug (doxycycline) being very strain dependent. The three drugs had different effects on susceptibility, with doxycycline generally having no effect, chloroquine generally increasing susceptibility and pyrvinium pamoate generally reducing susceptibility. The ability to simultaneously quantitate different aspects of microbial drug responses will facilitate the study of mechanisms of subpopulation responses in the presence of antimicrobial drugs.

**INTRODUCTION**

Microbial infections represent a tremendous threat to human health (Center for Disease Dynamics & Policy, 2015, and references within) and the increasing rate of antimicrobial resistance presents broad challenges that must be addressed. Understanding the biological basis of interactions between antimicrobials and microbes, and the processes by which resistant microbes arise and spread through a susceptible population, can guide the design of new strategies to reduce the appearance and spread of resistance. Treatment failure can arise due to infection with a non-susceptible (drug-resistant) organism that can grow robustly in the presence of a drug at a concentration that would normally kill or limit growth. Drug-resistant isolates can also appear during infection with microbes assessed as drug-susceptible due to complex interactions between the drug, the infecting microbial population and the host (Sanglard & Odds, 2002; Lee & Palsson, 2010). Furthermore, susceptibility testing may not capture the biological complexity of the drug response of some microbial isolates (Rex & Pfaller, 2002) and thus the ability to quantitate additional aspects of drug responses may help us understand the mechanisms by which non-susceptible strains survive in the presence of the drug.

**Abbreviations**: BMD, broth microdilution assay; CLSI, Clinical and Laboratory Standards Institute; MIC, minimum inhibitory concentration; OD, optical density.

Supplementary material is available with the online version of this paper.
The existence of persistent and recurrent infections despite theoretically lethal levels of the drug has long been observed (Bigger, 1944). The ability of subpopulations of cells to grow in a level of drug above their measured resistance level (at times termed tolerance, persistence, trailing growth, or heteroresistance) has been reported for bacteria, fungi and protozoa as well as for cancer cells (Cohen et al., 2013; Sharma et al., 2010). Here we use the term ‘tolerance’ to describe strains in which a subpopulation exhibits an epigenetic or physiological (and transient) ability to grow in supra-minimum inhibitory concentrations (MICs), i.e., phenotypic non-susceptibility yet genetic susceptibility.

To study tolerance, one must be able to measure it quantitatively. Standardized assays for quantitating drug susceptibility include the broth microdilution assay and the disk diffusion assay. Clinical standards that correlate microbial performance in these assays to clinical resistance have been determined for many pathogen–drug combinations in both North America (Clinical and Laboratory Standards Institute, CLSI, http://clsi.org/) and Europe (European Committee on Antimicrobial Susceptibility Testing, EUCAST). These assays are not designed to quantify tolerance (Lewis, 2010), and guidelines for their use explicitly recommend ignoring the biological signatures of tolerance (NCCLS, 2004). Accordingly, a subpopulation of tolerant cells that can grow at supra-MIC concentrations are often present and, occasionally, may result in incorrect assessments of susceptible strains as resistant (Matar et al., 2003).

Candida albicans is the fungal pathogen most prevalent in humans and the fourth most common hospital-acquired pathogen in the United States. Fluconazole, the primary antifungal used to treat candidiasis, is fungistatic: it inhibits fungal growth but does not kill the majority of the cells. Combination therapies, in which a second drug is used together with fluconazole, have been reported to increase the potential therapeutic efficacy of fluconazole (Liu et al., 2014, Butts & Krysan, 2012; Butts et al., 2013; Fiori & Van Dijck, 2012; Liu et al., 2014; Zeidler et al., 2013, and references within). However, the degree to which these different agents influence tolerance rather than resistance is usually not determined in a manner that permits quantitative comparison between different drug combination therapy regimes.

The goal of this study was to devise an assay that measures both resistance and tolerance to single drugs and to drug combinations, in a quantitative, reliable, reproducible and inexpensive manner. To that end, we developed diskImageR, an image analysis program that quantitates data from disk diffusion assays and measures both susceptibility and features of tolerant subpopulations. The disk diffusion assay is particularly amenable to this goal as it is adaptable for use with any drug, drug concentration or combination of drugs, and can be performed with diverse microorganisms, media, and incubation conditions in virtually any sterile microbial lab (Driscoll et al., 2012). Importantly, it is less labor intensive than broth microdilution assays (Fothergill, 2011; Pfäffer, 2012) and is, thus, better suited to studies that require the simultaneous assessment of many strains or multiple assay conditions. The diskImageR program removes user subjectivity by computationally assessing different drug response parameters. While this study focused on drug responses of C. albicans, diskImageR can be used to assess disk diffusion images from any pathogen/drug combination.

**METHODS**

*C. albicans* strains. Twenty-six diploid *C. albicans* clinical isolates that span the phylogenetic diversity of the species were chosen (Table 1). All strains were initially streaked onto YPD plates and grown for 48 h at 30 °C. A single colony from each strain was arbitrarily selected and frozen in 15% glycerol and stored at –80 °C for use in all assays.

**Disk diffusion assay.** The CLSI document M44 guidelines for antifungal disk diffusion susceptibility testing (NCCLS, 2004) were followed with slight modifications. In brief, strains were streaked from frozen culture onto Sabouraud agar and incubated for 24 h at 35 °C. Five to ten colonies were picked into 2 ml solution with 0.145 ml−1 saline and optical density (OD) was determined with a NanoDrop (ThermoScientific). OD was adjusted to 0.02 through either dilution with saline or by adding additional colonies, and 100 µl of diluted culture from each strain was plated onto Muller-Hinton plates and spread using sterile beads (3 mm, Fisher Scientific). After the plates had dried, a single 25 µg fluconazole disk (6 mm diameter, Becton, Dickinson and Company, USA) was placed in the centre of each plate. Plates were incubated at 35 °C for 24 h. To examine the influence of culture conditions on the assay, the same diluted strain cultures were also spread on YPD plates and incubated at 30 °C for 24 h with a 25 µg fluconazole disk in the centre of the plate. The experiment was replicated on three separate days. Twenty strains (1–20) were originally chosen blind with respect to resistance and tolerance and were predominantly susceptible. To conduct our assays on a broader range of strains we subsequently chose six additional strains (21–26) that were known to be intermediate or resistant. When assessing the second strain set on Muller-Hinton we found all strains (including a set of control strains) repeatedly yielded significant outlier values for tolerance, indicative of a media batch effect, and these data were not included. We conducted paired *t*-tests to assess the absolute difference between the two assay media on drug response parameters.

After 24 h of incubation, each plate was photographed individually. This step enables the laboratory component of the disk diffusion assay to be separated in time from parameter quantification, which facilitates analysis of large numbers of assays. The photography set-up and an example of an ideal photograph are presented in Fig. S1, available in the online Supplementary Material. Photographs should be taken at the highest possible resolution on a plain black background from a fixed distance directly overhead, all camera settings should be set manually (rather than automatically), and shadows on the plates should be minimized. The ‘best-practice’ disk diffusion experiments will have all strains for a particular experiment photographed on the same day. If this is not possible, a number of standard strains should be included for comparison of results from different days.

diskImageR package. diskImageR utilizes two freely available programs that can run on any computational platform: the statistical programming language R, (R Core Team 2014), and the ImageJ image analysis program (http://imagej.nih.gov/ij/). Although prior knowledge of R is helpful, it is not required, and the output of diskImageR can be analyzed in any program that can read text files (e.g., Microsoft Excel).
**Table 1. C. albicans** clinical strains; MIC and RAD\textsubscript{20} (radius of inhibition) were measured under CLSI conditions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Strain name</th>
<th>MTL genotype, patient status (if known), isolation site, country</th>
<th>MIC</th>
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zone diameter (NCCLS, 2004) by multiplying by 2 and adding the diameter of the diffusion disk (typically 6 mm).

FoG values are determined using the R function `rollmean`, which calculates the area under the curve in slices from the disk edge to each RAD cutoff (Fig. 1c, red hatch). This achieved growth is then compared to the potential growth, i.e., the area of the potential area of growth (dashed line, rectangle, shown here for FoG80). (d) A dataframe is created in R and saved as a CSV file, with one row for each photograph. (e) For replicate photographs, the average error across replicate photographs can be determined and saved as a dataframe in R and as a CSV file.

**Fig. 1.** diskImageR primary functions and workflow. Text in Courier font indicates the function call required from the user to initiate each step, all run in the R programming language. (a) The directory of disk diffusion assay plate photographs are specified by the user. With a custom macro, each photograph is automatically imported in turn into ImageJ. The pixel density is measured for 72 radii, every 5° from the centre of the disk. The trace for one such line is illustrated. (b) Custom R scripts calculate and plot the mean pixel intensity across all lines. (c) The background intensity of the plate is subtracted and a double logistic curve is fit to the data (thick black line). From the fit of the model, the RAD value is determined at the point where growth is reduced by 20% (RAD20, light blue dot), 50% (RAD50, blue) and 80% (RAD80, dark blue). FoG is calculated as the realized area of growth under the curve (red hatch) divided by the potential area of growth (dashed line, rectangle, shown here for FoG80). (d) A dataframe is created in R and saved as a CSV file, with one row for each photograph. (e) For replicate photographs, the average error across replicate photographs can be determined and saved as a dataframe in R and as a CSV file.
Reduced by ≥50% compared to the average of no-drug wells. A second OD reading after 72 h of incubation was used to measure trailing growth as the difference between MIC at 72 h and either 48 h or 24 h. The entire assay was performed twice for each environment, with two technical replicates for each strain measured each time; the reported values are the median of these four measurements. Kendall’s correlation tests (that do not assume bivariate normality of the data) were used to compare the mean results for each strain from the disk assay experiments and the median result from the liquid MIC experiments.

**Testing drug combinations.** A preliminary set of experiments with fluconazole (FLC) and doxycycline (DOX) was conducted on the standard lab strain (SC5314) to explore how best to visualize the interaction between the two drugs using the `diskImageR` framework. Doxycycline, an antibiotic, was recently reported to decrease the tolerance of *C. albicans* to fluconazole, in part because doxycycline altered the iron availability to cells (Fiori & Van Dijck, 2012). Doxycycline was demonstrated to reduce the proportion of the population that formed colonies and cleared the ZOI in an E-test strip assay. Lab strain SC5314 was streaked onto YPD from freezer stocks maintained at −80 °C and incubated at 30 °C for 24 h. Five colonies were picked into 1 ml PBS.

Optical density was read on an Infinite F200 PRO plate reader (Tecan) and standardized to 0.01; the cultures (200 µl per plate) were spread with sterile beads (5 mm, De-Groot, Israel) onto five 15 ml casitone plates (9 g/l BactoCasitone, 5 g/l yeast extract and 15 g/l Bacto agar) (Becton Dickinson) and 11.5 g/l sodium citrate dehydrate and 2 % glucose (Sigma-Aldrich). A single disk containing either 25 µg FLC (Gami-dor Diagnostics, Israel) or 50 µg DOX (Ghentham Life Sciences, UK) was placed in the centre of the control plates. We tested the use of both drugs in the same disk (25 µg FLC and 50 µg DOX) and the use of one drug in the disk (same concentrations as above) plus the other drug in the agar medium of the plate (1 µg ml−1 FLC or DOX 50 µg ml−1). The plates were incubated at 30 °C for 48 h and then analysed by `diskImageR`.

Two additional drugs were tested in combination with fluconazole on seven different strains chosen to broadly represent the diversity seen in the original strain set. Chloroquine (Chlo), an antimalarial drug, was recently found to be synergistic with fluconazole in six of nine fluconazole-resistant strains (but not in four fluconazole-susceptible strains) (Li et al., 2015). Pyrvinium pamoate (PP), an anti-parasitic drug used to treat pinworm infections, was identified in two *C. albicans* chemical screens: it was effective in preventing biofilm formation and inhibiting preformed biofilms (Siles et al., 2013) and was the top hit in a screen for drugs that differentially inhibit the growth of a fluconazole-resistant hit (5L) aneuploid strain compared to a wild-type euploid strain (Chen et al., 2015).

In all cases, FLC disks were used with the second drug mixed into 15 ml casitone medium in the petri plate. Preliminary tests with strain 17 (SC3514) of Dox, Chlo and PP were first tested with several 2-fold dilutions above and below the published values (Fiori & Van Dijck, 2012; Li et al., 2015; Chen et al., 2015). In all of these assays, FLC disks (25 µg) were placed on plates containing Dox, Chlo or PP, and final concentrations were chosen to optimize synergy with FLC (50 µg ml−1, 1 mg ml−1, 0.3 µg ml−1, respectively). The same final concentrations of Dox, Chlo or PP were used in broth microdilution assays.

**RESULTS AND DISCUSSION**

`diskImageR` was designed to provide an unbiased method to analyze disk diffusion assays, and to quantitate drug responses including RAD, the radius of inhibition, FoG, the fraction of growth achieved within the zone of inhibition (ZOI), and SLOPE, the rate of transition from no growth to full growth. The experiments described here examine the drug responses of *C. albicans* to fluconazole; `diskImageR` should be applicable though for most microbes and antimicrobial drugs.

**Among-strain variation in resistance and tolerance**

`diskImageR` was first used to examine the variation in fluconazole resistance among 26 clinical strains of *C. albicans*. The strains were assayed under two conditions: the CLSI guideline conditions used in clinical labs and standard rich medium YPD, often used in research labs. The resistance analysis here focused on RAD20 (20 % growth inhibition). This cutoff value is most consistent with the disk diffusion assay guidelines, i.e., what the average eye likely interprets as the point of prominent reduction in growth. Indeed, RAD20 correlates extremely well with the standard way of measuring the ZOI with a ruler (Fig. S2; Pearson correlation test, t2/σ=15.6, p<0.0001, cor=0.91).

The assay medium had a significant impact on drug susceptibility: strain replicates grown on YPD typically exhibited reduced susceptibility (smaller RAD) relative to replicates grown using the CLSI conditions (Fig. 2a, paired t-test; t2/σ=−8.32, p=0.0001). This is consistent with earlier reports that the assay medium influences the ZOI size. The majority of strains exhibited RAD values of 14–18 mm under CLSI conditions, which corresponds to a zone diameter of 34–42 mm, i.e., typical of susceptible strains (ZOI can be calculated by doubling RAD and adding the width of the disk, typically 6 mm). RAD was slightly decreased on YPD (2 mm on average, Fig. 2a), yet this did not change the interpretation of drug susceptibility (NCCLS, 2004) and there was a significant correlation between RAD measured under CLSI or YPD (Kendall’s tau correlation test, z=3.39, p<0.0007, cor=0.62; Fig. S4a).

For measuring tolerance, we chose to focus on FoG50 because it maximizes differences among strains; similar results were obtained for the other FoG values (Fig. S3). The assay medium had a more dramatic and less predictable effect on FoG50 than RAD. Strains assayed on YPD tended to have increased tolerance relative to CLSI conditions (Fig. 2a, paired t-tests; FoG50: t2/σ=8.01, p<0.0001; SLOPE: t2/σ=4.80, p=0.0001). On average there was a 0.30 (30 %) increase in FoG50 assayed on YPD compared to CLSI, yet the fraction of tolerant cells was more than double this difference in some strains (Fig. 2b); because of the different ways that the assay medium influenced FoG in different strain backgrounds, there was no significant correlation between FoG50 in the two environments (Kendall’s tau correlation test, z=1.35, p=0.24, Fig. S4b). The difference in SLOPE among the strains was intermediate to differences seen for RAD and FoG. Although the influence of the environment was more strain-dependent for SLOPE than RAD (Fig. S4c), there was a significant correlation between SLOPE assayed on YPD compared to CLSI (Kendall’s tau correlation test, z=1.98, p=0.048, cor=0.32), with strains on average ~1.6 times more sensitive on CLSI conditions than YPD (though with a large range of variation between

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0.8 and 2.8 times more sensitive). These results likely reflect diversity in the mechanisms of fluconazole tolerance across the diverse set of C. albicans strains tested.

RAD and FoG values did not correlate in either set of culture conditions (Kendall’s tau correlation tests; CLSI: \( z = 0.78, p = 0.43 \); YPD: \( z = 0.27, p = 0.79 \)). Thus, RAD and FoG represent different biological properties involved in how the growth of microbes is affected by antimicrobial drugs. SLOPE did not correlate with RAD in CLSI conditions, but did correlate with RAD in YPD (CLSI: \( z = -0.17, p = 0.87 \); YPD: \( z = 2.38, p = 0.02, \text{cor}=0.35 \)). By contrast, FoG and SLOPE were strongly correlated with each other in both sets of conditions (CLSI: \( z = -2.56, p = 0.01, \text{cor}=0.44 \); YPD: \( z = -4.50, p<0.0001, \text{cor}=0.68 \)), implying that SLOPE and FoG likely calculate related aspects of tolerance in different ways. Thus, we focused on RAD and FoG going forward, as representative parameters for the two different drug response properties.

**Correlation between disk diffusion and broth microdilution assays**

We next compared results from diskImageR with those from broth microdilution assays (BMD). As expected, a significant correlation was found between \( \text{RAD}_{20} \) and \( \log_{10}(\text{MIC}_{50}) \) for both CLSI and YPD conditions (CLSI: \( z = -3.42, p=0.0006, \text{cor}=-0.51 \); YPD: \( z = -4.89, p<0.0001, \text{cor}=-0.68 \)).
A significant correlation has been reported previously for disk diffusion and BMD assays for many species/drug combinations (e.g. Berghaus et al., 2014; Coorevits et al., 2015; Matar et al., 2003).

For some isolates, the ability of a subpopulation to grow above the MIC was apparent in both the disk diffusion (quantified by FoG) and the BMD assays (quantified as trailing growth—the difference in MIC between growth at 72 h and growth at 24 or 48 h). Interestingly, FoG and trailing growth were not correlated for assays performed using the CLSI or YPD conditions (CLSI: $z=-0.95$, $p=0.34$; YPD: $z=0.15$, $p=0.88$, Fig. 4). This suggests that FoG and trailing growth capture different aspects of the tolerance phenomenon. Of note, FoG measures the growth of individual colonies in the population, while trailing growth involves an explicit time component and measures the behaviour of a population of cells.

**RAD can be used to infer MIC**

Motivated by the correlation between RAD and MIC, we asked if the relationship between them is primarily based on diffusion of the drug in the plate. We used the equation for diffusion in two dimensions to approximate the drug concentration profile along the plate. With this equation an MIC value can be estimated from a series of experiments using different drug concentrations in the disk, even if the actual diffusion rate of the drug in the plate is unknown.

**Fig. 3.** Significant correlation between RAD calculated by *diskImageR* and log$_2$(MIC$_{50}$) from broth microdilution. A significant correlation between the two assays is observed whether strains are measured in (a) CLSI conditions or (b) YPD. (c) Data for 2100 published ZOI measurements (Barry et al. (2002, Fig. 1) and Pfaller et al. (2003, Fig. 1) were plotted as log$_2$(MIC$_{50}$) vs RAD$^2$. ZOI values were converted to RAD (by subtracting 6, the width of the disk, and dividing by 2) and the average RAD value was determined for each MIC value to avoid skewing the relationship by the overabundance of sensitive strains in the dataset. This provided a standard curve for determining MIC values from RAD.

**Fig. 4.** No significant correlation between FoG$_{50}$ in disk diffusion assays calculated by *diskImageR* and trailing growth from broth microdilution assays. Similar results were obtained using (a) CLSI conditions or (b) YPD conditions.
(for calculation details see Supplementary File S3). In some species and drugs, the relationship between log$_2$(MIC) and RAD is quadratic, while in others it is linear (Bonev, 2008), reflecting other processes, in addition to diffusion, that change the drug concentration profile (such as advection or degradation). When the approximated values of MIC obtained by calculating the diffusion profile were compared with the MIC values obtained by BMD, we found that the quadratic model results were in good agreement with the BMD results (File S3, Table S1). This result supports the hypothesis that diffusion is the major mechanism driving the disk diffusion assays (Bednár, 2000).

Given the above result, we can take advantage of this quadratic relationship between RAD and log$_2$(MIC) to infer MIC values for strains with known RAD. We obtained two datasets from the literature that included ~2100 measurements of the influence of fluconazole on the Candida species of MIC data from BMD and ZOI data from disk diffusion assays (Barry et al., 2002; Pfaller et al., 2003). We converted ZOI to RAD and regressed log$_2$(MIC) on RAD$^2$ to determine the slope and intercept (Fig. 3c). This relationship matched well with the parameters estimated using CLSI conditions or YPD (Fig. 3) and with diskImageR analysis of RAD on plates using other media, such as casitone (data not shown). Thus, the MIC of a new strain can be determined from these parameter estimates using the measured RAD value; this calculation is embedded in the diskImageR package. Within diskImageR the user can either use data already provided from the literature for a number of species/drug combinations (Table S2) or provide a set of RAD and MIC values to generate a new calibration curve for any specific organism and drug combination.

**diskImageR can distinguish the contributions of drug combinations to RAD and FoG**

We next compared the effects of three different drug combinations reported to synergize with fluconazole against C. albicans. The effect of doxycycline (DOX), chloroquine (Chlo) and pyrvinium pamoate (PP) on seven C. albicans strains was tested by using a 25 µg ml$^{-1}$ fluconazole disk on the plate and adding the second drug to the medium (preliminary results were similar regardless of how drugs were distributed, Fig. S5). The three drugs had different influences on RAD and FoG (Fig. 5a). Doxycycline did not affect RAD, regardless of the strain’s background (Fig. 5a), consistent with a previous report (Fiori & Van Dijck, 2012). By contrast, PP caused decreased susceptibility to fluconazole in fluconazole-susceptible strains (decreased RAD), while chloroquine increased susceptibility (increased RAD) in fluconazole-susceptible strains. The drugs did not influence

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**Fig. 5.** Second drugs in combination with fluconazole influence MIC, RAD and/or FoG. (a) RAD$_{20}$ and (b) FoG$_{50}$ were measured for strains plated with a 25 µg fluconazole disk on casitone plates alone (FLC, black circles) and with doxycycline (DOX, open circles), pyrvinium pamoate (PP, squares), or chloroquine (Chlo, diamonds) in the plate. The three drug combinations tended to decrease FoG$_{50}$ relative to fluconazole alone, though the influence of DOX was dependent on strain background; PP increased RAD$_{20}$ while DOX and Chlo showed no changes or small reductions in RAD$_{20}$. (c) The same strains and drug combinations were also assessed for resistance with a broth microdilution test.
strain 12, which was fluconazole resistant (non-susceptible) initially, and they had a minimal effect on strains 1 and 18, which were also initially more resistant than other strains.

Importantly, each of the three drugs tended to reduce FoG in combination with fluconazole, and the magnitude of the effect was different for different strains. For PP and Chlo, the addition of the second drug reduced FoG to similar low levels in all strains, regardless of initial strain tolerance (Fig. 5b). By contrast, although the effect of DOX also tended to reduce FoG, in certain strain backgrounds this effect was minimal or even reversed. These results illustrate how diskl mageR facilitates quantification of the contributions of different mechanistic aspects of combinatorial drug responses. They also highlight the importance of analysing drug effects in different strain backgrounds. Furthermore, the effect of these drugs on different aspects of growth in drug was not as readily distinguishable in CLSI BMD assays (Fig. 5c).

Conclusions

diskImageR provides a theoretically unbiased method to quantify disk diffusion assays for any microbial species/microbial drug combination. In addition, it distinguishes between drug susceptibility, which is a function of drug concentration, and two other correlated parameters, FoG and SLOPE, which reflect the ability of a subpopulation to grow at supra-MIC levels. diskImageR can compare drug responses across strain backgrounds and incubation conditions, with different strains maintaining their respective rank order under most assay conditions. For a broad range of C. albicans strains on fluconazole, the standard CLSI conditions revealed higher susceptibility (higher RAD/lower MIC) and less tolerance (FoG) relative to YPD conditions. In general, the assay condition used had a more predictable influence on resistance (RAD) than tolerance (measured as either FoG or SLOPE). Plain diffusion is the driving force that determines the ZOI in the disk diffusion assay for C. albicans with fluconazole, allowing this relationship to be used to infer MIC values from RAD. Finally, diskImageR analysis of different drug combinations with fluconazole revealed insights into the distinct effects of each second drug on both RAD and FoG. This ability to quantitate different aspects of drug response in a single simple assay will aid in future studies that aim to identify the mechanisms of drug responses including, but not limited to, resistance.

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