Using a variant of the optomotor response as a visual defect detection assay in zebrafish

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ABSTRACT

We describe a visual stimulus that can be used with both larval and adult zebrafish (Danio rerio). This protocol is a modification of a standard visual behavior analysis, the optomotor response (OMR). The OMR is often used to determine the spatial response or to detect directional visuomotor deficiencies. An OMR can be generated using a high contrast grated pattern, typically vertical bars. The spatial sensitivity is measured by detection and response to a change in grating bar width and is reported in cycles per degree (CPD). This test has been used extensively with zebrafish larvae and adults to identify visual- and/or motor-based mutations. Historically, when tested in adults, the grated pattern was presented from a vertical perspective, using a rotating cylinder around a holding tank, allowing the grating to be seen solely from the sides and front of the organism. In contrast, OMRs in zebrafish larvae are elicited using a stimulus projected below the fish. This difference in methodology means that two different experimental set-ups are required: one for adults and one for larvae. Our visual stimulus modifies the stimulation format so that a single OMR stimulus, suitable for use with both adults and larvae, is being presented underneath the fish. Analysis of visuomotor responses using this method does not require costly behavioral tracking software and, using a single behavioral paradigm, allows the observer to rapidly determine visual spatial response in both zebrafish larvae and adults.

Keywords: behavioral neuroscience, optomotor response, spatial frequency, visuomotor, zebrafish

BACKGROUND

Zebrafish (Danio rerio) are an excellent animal model for functional studies due to their short life cycle, sequenced genome, and commercially available mutant strains [1]. To facilitate use of this model in high throughput studies, methods for functional assessment must be highly reliable, repeatable, quantifiable, rapid, and inexpensive. One organ system that lends itself to functional studies via these throughput mechanisms is the visual system. In zebrafish, tests utilizing the visual sensory system are well established, as is the associated anatomy, partially because of the heavy reliance on their visual system for optimal fitness [2]. Behavioral assays of vision are not only applicable to studies of retinal pathology but also provide a whole-animal context of neural and behavioral function determined at the cellular level [3,4].

The optomotor response (OMR) is an innate visuomotor reflex characterized by the fish swimming in the same direction as a high-contrast visual stimulus. The swimming movement helps to stabilize the fish's position with respect to the stimulus. The OMR is a variation of the optokinetic response (OKR), another vision-based behavioral response which has been used in a large group of taxa, including humans [5]. Both OMR and OKR are used with zebrafish. The OMR is well characterized in larval zebrafish as a screen for aberrant motion detection

during development [1,6]. These results are often coupled to the OKR experiments, which identify retina-based defects. Because adults are difficult to immobilize, which is a requirement for the OKR, the OMR is often used to assess changes in vision-based behaviors. The use of the OMR task in adult zebrafish has identified visual impairments due to genetic mutations and toxin exposures [7,8]. Zebrafish OMR is generated by several separate visual and motor circuits, many of which overlap with the circuits activated by the OKR in the eyes [9]. OMR neural circuitry is evolutionarily conserved, involving binocular neural activation, making the OMR separate from the OKR [10]. This suggests additional pretectal modulation is directly related to motor tuning via reticulospinal cells [11]. Thus, defects in the OMR may suggest a major visuomotor defect that may not be detected by other behavioral analyses. Though many studies have examined visuomotor capacity and behavioral acuity in zebrafish, the methods are based on those validated for larval zebrafish, which may not work as well for adults. This has been demonstrated by the need for slightly different OMR stimulus methods required for two recent publications from our lab [8,12]. The method described here has been altered from those initial works to address that issue. The result demonstrates a novel and inexpensive OMR assay that quantitatively assesses visuomotor and behavioral spatial responses in both larval and adult zebrafish.

MATERIALS

Animals

Wild-type adult zebrafish (Danio rerio) obtained from either the existing colony at American University or, if needed, a commercial supplier (LiveAquaria, CA, United States of America) and their offspring were used. Fish were housed in an aquatic facility (AHAB-Pentair),

maintained at 28 ± 3°C with a 14:10 light-dark cycle. The fish were fed Tetramin flakes supplemented with either powdered (larvae) or live (adults) brine shrimp twice daily [13].

To obtain larvae, zebrafish were bred in-house following established protocols. In brief, group breeding was performed by placing multiple female and male adult zebrafish from the breeding colony in a breeding chamber overnight to ensure genetic variability of the wildtype offspring. The next morning, 30–60 min after lights on, embryos were collected, staged (shield to 75% epiboly), and placed in petri dishes maintained at the same water temperature and light cycle as adults. Larvae were housed in 100 mm petri dishes in a temperature-controlled incubator (Heratherm), at the same environmental conditions as stock tanks, for at least two weeks prior to being shifted into the aquatic facility. Dishes were checked daily to remove debris and feed the larvae.

All experiments were performed within the protocols and guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of American University.

Equipment

- ✓ PsychoPy Psychophysics code package [14]
- ✓ Python-based stimulus code (source code available at github. com/MattLeFauve/OMRProject)
- ✓ Computer capable of running PsychoPy
- ✓ Standard Computer Monitor
- ✓ Larval Petri dish (100 mm diameter)
- ✓ Adult Dish (28 cm diameter)
- ✓ Video Recorder (Canon FS40 Handheld was used in this study)
- ✓ Closed Door Behavior Chamber/Cabinet (optional)
- ✓ Video playback software (VideoLan VLC Mediaplayer was used in this study)
- ✓ Repeating stopwatch

PROCEDURE

Assembling the OMR setup

Figure 1 shows the OMR Task setup.

Running the OMR setup

1. Install Psychopy on your computer (http://psychopy.org/installation.html).

2. Download the stimulus from GitHub. Please see **Table 1** to determine how to modify the stimulus if necessary for your experimental setup.

3. Connect the monitor and the computer and test that the stimulus successfully fills the monitor screen. Prior to playing the stimulus, the monitor should be calibrated using the tools provided on a Windows or Mac computer (https://www.digitaltrends.com/computing/how-to-calibrate-your-monitor/). This will ensure the stimulus is presented at the optimal optical resolution.

4. Place a 20.32 × 25.4 cm (8 in × 10 in) transparent film/acetate on the monitor to prevent water damage. Prior to recordings, run the stimulus and position the experimental dish on the monitor to determine the optimal location for both the adult and larval dishes. The dish should be directly above the stimulus and positioned so the convergent point for the pinwheel is in the center of the dish.

5. Once the optimal dish position is determined, trace the outline of the bowl or make other markings, so you can consistently position the dish in the same location between trials.

6. Position the camera above the monitor, inside the behavior chamber (**Fig. 1**). Camera height is somewhat arbitrary (in our case ~45.72–60.96 cm above the dish) but should be high enough to allow movement/

transferring of bowls and fish below it, but low enough so that the experimental dish fills the video screen. Recording within a behavioral chamber is optimal, as it prevents distraction of the fish and also allows control of external lighting while taking videos.

7. To prevent monocular visual distraction for the fish during stimulus presentation, place non-reflective tape around the outside of the bowl to the water line.

8. Place a physical barrier such as a small straight sided glass container over the central portion of the testing dish to prevent visual distraction at the point source of the stimulus as demonstrated in **Figure 1B**. To prevent visual distraction stemming from this barrier, the center barrier should also be covered with non-reflective tape. This provides a swimming arena for the fish that is an annulus, so the fish swims either clockwise or counterclockwise within the dish.
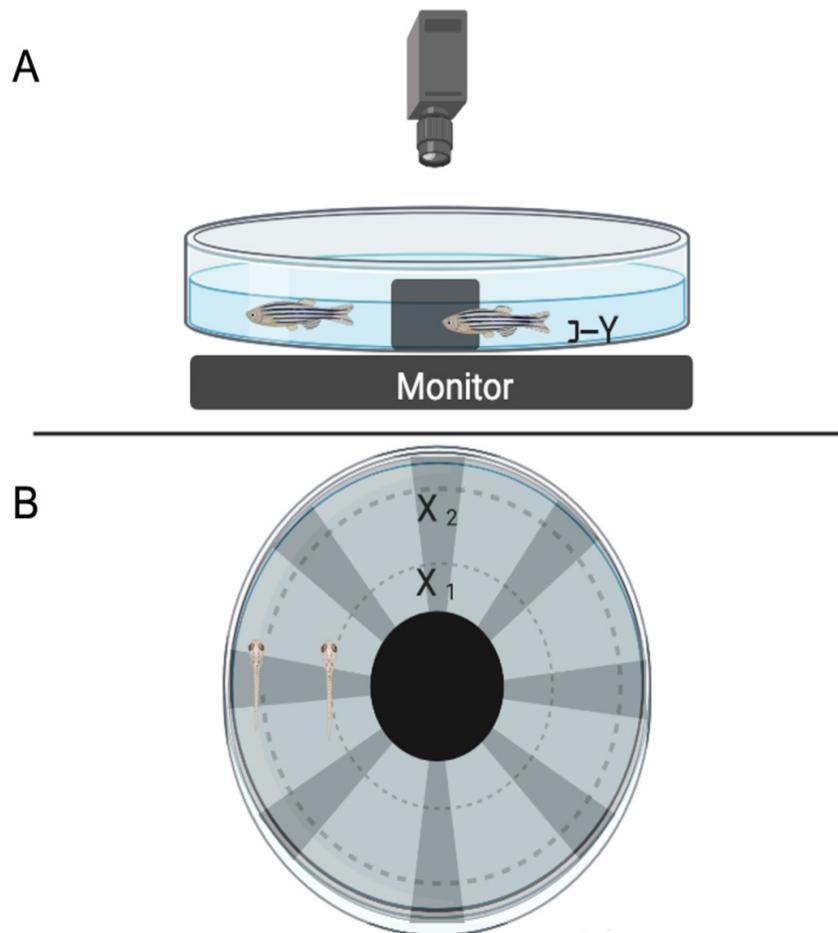
Figure 1. Experimental setup. A. Setup from the side. **B.** Setup from the top, with the stimulus running. The computer monitor displaying the stimulus is covered with a transparent 20.32 × 25.4 cm (8 × 10 in) piece of acetate to prevent water damage to the monitor. The dish should always be situated in the center of the monitor, directly above the stimulus. Camera height above the dish is shown as 45.72–60.96 cm (~18–24 in) but should be adjusted to allow detailed viewing of the entire dish and the movement of the fish in the dish. X_1, X_2, and Y are measurement parameters used to calculate visual angle (see text step 22).

Presentation of the OMR

9. Fill the trial dish ~2/3 full with fresh system water. The larvae should be in 100 mm diameter dishes and the adults should be in 28 cm diameter dishes (see equipment list).

10. Place up to 10 larvae or 1–2 adult fish in the dish. Position the glass container (step 8 above) in the center of the dish.

11. Transfer the dish containing fish into the behavioral chamber and position it on the monitor, taking care to make sure it is in the center of the monitor and aligned with the position markers (steps 4–5 above) on the

transparent cover. Allow the fish at least three minutes to acclimate to the testing chamber. Acclimation to the dish is performed within the recording chamber.

12. Turn the camera on and be sure the fish are visible.

13. Begin recording. Following the directions in the "OMR_Stimulus" code, project the stimulus below the fish. The stimulus is designed to rotate clockwise then counterclockwise with a blank gray screen in the middle. The default timing is 30 s for each direction and the blank screen, but timing can be altered by changing the second number in the TIME line. Altering the TIME line number will change the seconds of each stimulus direction, including the blank screen. The provided stimulus code has been written at the optimal speed and angular cycles to elicit an optimal response for each age group. The optimal speed and angular cycle number are presented in **Table 1**.

14. After allowing the stimulus to make as many full rounds of clockwise-gray-counterclockwise as necessary, stop the stimulus by pressing the spacebar and stop the video recording. Zebrafish tend to exhaust and need swimming recovery time after three to four full rounds of the stimulus when presented at 20 s intervals.

15. Once the behavior has been recorded, return fish back to the holding/stock tanks.

16. 16. This process can be repeated as many times as necessary to behaviorally test each fish. Extinction of the OMR is possible however, so individuals should not be tested with more than 10 full rounds (clockwise, gray, counterclockwise) in a 48 h period.

Table 1. Stimulus line descriptions.

Line	Description	Typical ranges
Time	Seconds that the stimulus and blank "recovery" screen are presented	30–60 s is the typical presentation time that reduces the potential for rapid exhaustion
Speed	Time (in seconds) that it takes for one angular cycle to go one full revolution. Number = rad/s	Larvae Stimulus: 1.04 Adult Stimulus: 1.033
Grating (angular cycles)	Number of angular cycles presented	Prime larval response: 16 angular cycles Prime adult response: 12 angular cycles Spatial resolution analysis tested 2–64 angular cycles
Grating (contrast)	Strength of the leading edges of the stimulus	The best OMRs were elicited by strong leading edges (0.9–1.0) (LeFauve, 2015, personal observation) We do not recommend changing this parameter in the stimulus code

Analysis of the video, detection assessment for visuomotor defect discovery

17. Ensure fish are visible in the recorded video before proceeding with data analysis. Adjustment of the camera position may be necessary.

18. Movement of the fish in response to each stimulus can be analyzed in three ways, described below. In general, as this method is designed to be implemented easily without costly behavioral software or time-costly coding setup, it is not well-suited to using currently available open-source computer vision tracking software such as PathtrackR [15].

   18.1. Scan sampling—This can be applied to both larvae and adults but works best for larvae as the size of the Petri dish may preclude the larvae from making a full revolution during stimulus presentation. Scan sampling behavioral analysis can be used to assess group behaviors quickly. To assess OMR success, at 10 s intervals, perform a rapid clockwise sweep of the dish and count the individuals moving in the direction of the stimulus. All individuals moving with the direction of the stimulus at the time of the sweep are to be counted as demonstrating a positive OMR [16]. Individuals not moving are therefore not showing an OMR and individuals moving in the opposite direction are showing a negative OMR. For ease of analysis, this study combined individuals showing no OMR and showing a negative OMR into a "non-positive OMR" group. If comparing treatment groups, it may be helpful to count the number of larvae moving during the break period when the stimulus is not being presented. Zebrafish have been shown to have motion aftereffect and so the first sweep of fish should not be counted using this method.

18.2. Counting the number of full revolutions—Adults are able to swim completely around the dish without exhaustion during the stimulus interval, so the number of full revolutions can be counted. Counting the number of times each fish makes one full revolution around the dish during the stimulus presentation is similar to counting individuals following the OMR stimulus when presented in a unidirectional pattern. This method works well for adult stimulus presentations.

18.3. Combination of observation and computer tracking. Open source behavioral software may be a viable option to track the fish in this behavioral setup. Easier to use options that the authors recommend include PathtrackR [15], ZebraZoom [17]. Current open-source behavioral tracking software performs better when there is a constant background as most computer vision-based tracking is done using organism-background contrast differences. As a result, a high contrast pinwheel stimulus, presented below the fish, may result in automated tracking errors, as a dark fish above a dark portion of the stimulus would be 'lost' and not counted. Thus, the method described here may lend itself better to observer-based video tracking rather than computer-based tracking.

Analysis of the video, spatial frequency analysis

19. If necessary, repeat "Presentation of the OMR". It is possible (and necessary when recording responses in larval *vs.* adult fish) to change the number of angular cycles presented to the fish, explanation of this can be found in **Table 2**. Angular cycles presented can be altered by changing the "angular cycles" number in the "grating" lines. Up to three angular cycle amounts can be tested in one recording session before the zebrafish will stop responding. If more angular cycle tests are needed for an individual, recovery time after bout swimming can take up to 15 min (LeFauve, 2015, personal observation). Recovery can be conducted in the testing apparatus with the monitor turned off. As stated above, zebrafish should not be tested with more than 10 full rounds (clockwise-gray-counterclockwise) in a 48 h period.

20. Repeat step 17.

21. Movement of the fish in response to each angular cycle number can be done using the same measurements provided in step 18. This will generate an optimal response curve (**Fig. 2B**). The optimal response curve indicates what angular cycle demonstrates the strongest response based on either the number of individuals exhibiting a positive OMR or the number of revolutions made by each individual in a treatment group.

22. The results are the proportion of time fish swim in one direction *vs.* either the frequency of grating (the number of angular cycles presented, as in **Fig. 2B**) or the visual angle subtended by one cycle of the grating on the fish retina. The visual angle can be calculated by the arctan of the distance from the fish to the screen (Y in **Fig. 1A**) divided by the distance covered by one angular cycle. In our example, the distance from the fish to screen was 40 mm for adults and 10 mm for larvae, the distance of the cycle depends on the fish's radial position in the dish (as in the X_1 and X_2 values in **Fig. 1B**). The width of the angular cycles can be calculated by dish circumference divided by the number of angular cycles.

APPLICATION AND VALIDATION

We have optimized a variation of the standard OMR assay that is suitable for use with both larval and adult zebrafish. Previous work demonstrated that an OMR can first be detected at 5 dpf and reliably demonstrated by 7 dpf [18]. While the stimulus elicits an OMR beginning at 7 dpf, it becomes considerably more robust with age, as evidenced by the responses at 10 dpf and 13 dpf when compared to a blank lit background screen (*t*-test, $P < 0.001$) (**Fig. 2A**). Maaswinkel and Li suggested the optimal speed of the stimulus was 103 deg/s for juvenile to adult zebrafish [19]. To generate a comparable method, we maintained the stimulus at that speed while different spatial frequencies were tested. Larval zebrafish response peaked at 16 angular cycles and adult zebrafish response peaked at 12 angular cycles (**Fig. 2B**). For longer duration studies, there appears to be a developmental time point when the zebrafish stop responding to the larval stimulus and begin responding to the adult stimulus. This occurs at 22–23 dpf and may reflect changes in the visual system at these ages (**Fig. 2C**).

The classic OMR is used for visuomotor defect detection in a variety of taxa, including Drosophila, zebrafish, rodents and humans [20]. These designs are all similar, with most stimuli presented as a rotating drum around the test subject. This type of experimental apparatus can present a myriad of challenges stemming from costly experimental setups to high analysis time. The method described here reduces the experimental cost and demonstrates an easy and repeatable observation technique by presenting the rotating drum stimulus as a computer-generated rotating pinwheel that is projected below the animal. Though this technique of presenting stimuli from below is currently used with zebrafish larvae, it is not commonly used with adults, which often require an OMR setup with the stimulus presented from the side [1,21]. Thus, current OMR analysis across the life span of the fish requires two different laboratory set-ups. With our technique, however, only one experimental set-up is needed, as both larvae and adults respond to the computer-generated stimulus. The circular nature of our OMR technique reveals that adult zebrafish are capable of responding to an OMR stimulus presented from below, but only when side vision is obscured.

Previously, we used variations of this technique in our lab to identify changes in the zebrafish visual system in response to developmental

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exposure to heavy metals [12] and endocrine disruptors [8]. The stimulus used in these initial studies has been further modified to reduce any startle response exhibited by the fish during stimulus presentation changes and the overall luminosity of the stimulus while maintaining angular cycle

contrast. We have also identified the age at which stimulus parameters change for a larval to adult zebrafish transition. Additional work needs to be done to determine how that age transition, and resulting behavioral response change, plays a role in organism function.
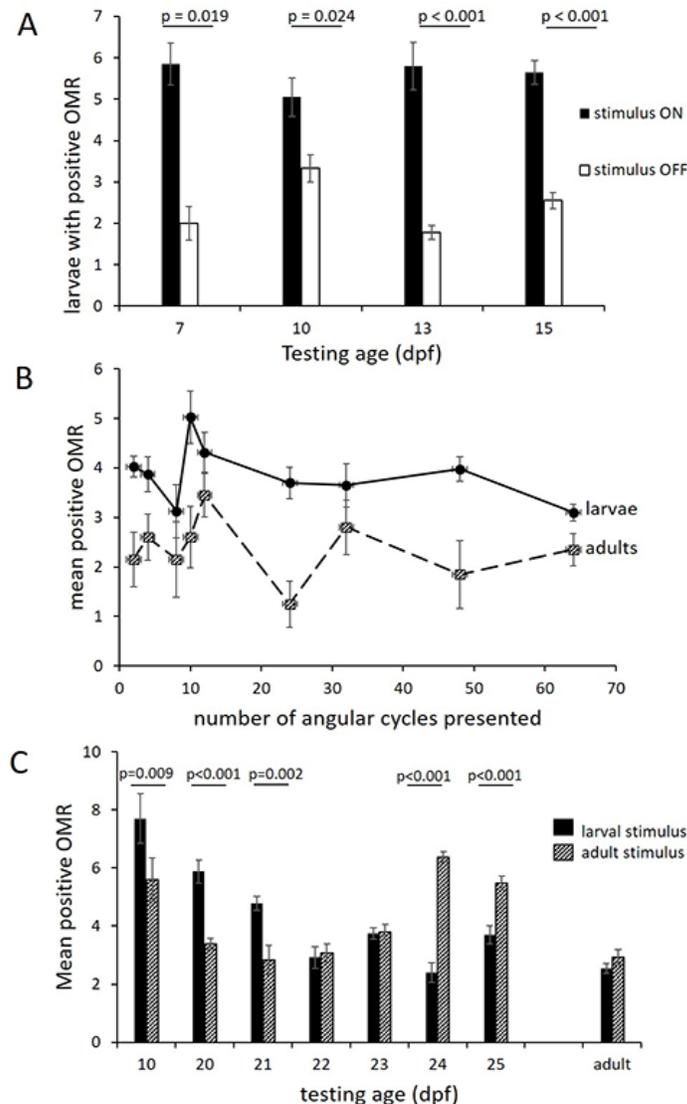
Figure 2. Positive OMR responses in larval and adult zebrafish. A. Stimulus-elicited positive OMR in larval zebrafish. This is the mean number (± standard error = 20–30 per testing age) of larvae displaying a positive OMR when the stimulus is presented (black bars) *vs.* during the gray resting screen (white bars). **B.** Positive OMRs evoked in response to different numbers of angular cycles. Mean number (± standard error) of larval ($n = 20$ per stimulus) and adult ($n = 6$ per stimulus) zebrafish demonstrating a positive OMR during stimulus presentation. Responses were recorded across a range of spatial frequencies, represented by an increasing number of angular cycles in the stimulus. **C.** Mean stimulus responses across ages tested. Larval stimulus presentation (black bars) resulted in the largest number of positive OMR in zebrafish aged 21 dpf or younger. Conversely, the adult stimulus presentation (hashed bars) resulted in the largest number of positive OMRs in zebrafish aged 24 dpf and older. At intermediate ages (22–23 dpf) zebrafish responded similarly to both stimulus types, suggesting a transition stage. Statistical differences at each age (in **A** and **C**) were assessed using a *t*-test (IBM SPSS, ver. 27). ($n = 10$–26 per testing age) Significant differences are indicated by bars, with corresponding *P*-values. α = 0.05.

In summary, we have modified an optomotor technique that is easily adaptable for use with adult and larval ages in the zebrafish model. The ability to detect changes across the lifespan, using the same method in both larval and adult zebrafish, will undoubtedly prove valuable.

TROUBLESHOOTING

This method is moderately straightforward in application, but issues can potentially arise in the stimulus code. Presented in **Table 1** are descriptions of each line that a user can alter, ultimately changing

a component of the stimulus to be presented to the fish. Commonly encountered issues not already discussed are presented in **Table 2**.

Table 2. Troubleshooting.

Step number	Problem	Causes	Suggestions
"Running OMR Setup"	Fish are jumping out of the OMR bowl	Fish may be startled by the light source below them	Letting the fish acclimate to the OMR setup may eliminate this problem, and after acclimation, the authors did not experience fish leaving the testing chambers. If this does not solve the issue, placing a clear piece of acrylic on top of the testing container may be necessary.
14	Larvae are remaining active during the blank "control" period of the stimulus presentation	Fish are impacted by motion aftereffect motion as elicited by this stimulus	Do not count the number of larvae swimming during the first 10 s scan sample interval to allow them time to overcome the visual illusion of motion aftereffect OR only count the fish locomotion while the stimulus is being presented.
15	Testing visual acuity with variable angular cycle amounts, but needing speed to be consistent	Increased angular cycle amount will result in the stimulus increasing in speed based on stimulus code. This can be accounted for by changing the "SPEED" line.	Below are the numbers to insert into the "SPEED" line for given angular cycles: • 2 angular cycles = 1.005 • 4 angular cycles = 1.01 • 8 angular cycles = 1.02 • 12 angular cycles (validated adult stimulus) = 1.033 • 16 angular cycles (validated larval stimulus) = 1.04 • 20 angular cycles = 1.05 • 24 angular cycles = 1.06 • 30 angular cycles = 1.068 • 36 angular cycles = 1.082 • 40 angular cycles = 1.095 • 46 angular cycles = 1.115 • 50 angular cycles = 1.12 • 56 angular cycles = 1.133 • 60 angular cycles = 1.143 • 66 angular cycles = 1.15

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