Integrating transient heterogeneity of non-photochemical quenching in shade-grown heterobaric leaves of avocado (Persea americana L.): Responses to CO2 concentration, stomatal occlusion, dehydration and relative humidity

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Integrating transient heterogeneity of non-photochemical quenching in shade-grown heterobaric leaves of avocado (Persea americana L.): Responses to CO2 concentration, stomatal occlusion, dehydration and relative humidity

Abstract
Long-lived shade leaves of avocado had extremely low rates of photosynthesis. Gas exchange measurements of photosynthesis were of limited use, so we resorted to Chl fluorescence imaging (CFI) and spot measurements to evaluate photosynthetic electron transport rates (ETRs) and non-photochemical quenching (NPQ). Imaging revealed a remarkable transient heterogeneity of NPQ during photosynthetic induction in these hypostomatous, heterobaric leaves, but was adequately integrated by spot measurements, despite long-lasting artifacts from repeated saturating flashes during assays. Major veins (mid-vein, first- and second-order veins) defined areas of more static large-scale heterogeneous NPQ, with more dynamic small-scale heterogeneity most strongly expressed in mesophyll cells between third- and fourth-order veins. Both responded to external CO2 concentration ([CO2]), occlusion of stomata with VaselineTM, leaf dehydration and relative humidity (RH). We interpreted these responses in terms of independent behavior of stomata in adjacent areoles that was largely expressed through CO2-limited photosynthesis. Heterogeneity was most pronounced and prolonged in the absence of net CO2 fixation in 100 p.p.m. [CO2] when respiratory and photorespiratory CO2 cycling constrained the inferred ETR to -75% of values in 400 or 700 p.p.m. [CO2]. Likewise, sustained higher NPQ under VaselineTM, after dehydration or at low RH, also restricted ETR to -75% of control values. Low NPQ in chloroplast-containing cells adjacent to major veins but remote from stomata suggested internal sources of high [CO2] in these tissues.

Keywords
heterogeneity, occlusion, stomatal, concentration, co2, dehydration, relative, responses, l, humidity, americana, integrating, persea, avocado, leaves, heterobaric, grown, shade, transient, quenching, photochemical, non

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Title. Imaging Transient Heterogeneity in Non-photochemical Quenching in Shade-grown Leaves of Avocado (*Persea americana* L.) in Response to CO₂ concentration, Relative Humidity and Dehydration.

Running head. Transient heterogeneity of NPQ in avocado leaves

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Title. Imaging Transient Heterogeneity in Non-photochemical Quenching in Heterobaric Shade Leaves of Avocado (Persea americana L.): Responses to CO₂ concentration, Relative Humidity and Dehydration

Running head. Transient heterogeneity of NPQ in avocado leaves

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Abbreviations:
A: antheraxanthin
CFI: Chlorophyll fluorescence imaging
[Cₐ]: ambient CO₂ concentration
[Cᵢ]: intercellular CO₂ concentration
[CO₂]: CO₂ concentration
DESV: de-epoxidation status of the violaxanthin cycle
ΔpH: putative transthylakoid pH gradient in chloroplasts

ETR: Photosynthetic electron transfer rate

L: lutein

NPQ: Non-photochemical quenching of chlorophyll fluorescence

NPQ_{ΔpH}: NPQ in the absence of de-epoxidation of xanthophyll pigments

NPQ_{AZ}: NPQ after de-epoxidation of violaxanthin

Lx: lutein epoxide

V: violaxanthin

Z: zeaxanthin
Abstract
Long-lived shade leaves of avocado and some other tropical trees have extremely low rates of photosynthesis. This paper demonstrates the limited usefulness of photosynthetic gas exchange in these leaves and the efficacy of chlorophyll fluorescence imaging (CFI) for evaluation of non-photochemical quenching (NPQ) and rates of photosynthetic electron transport (ETR). It revealed a remarkable transient heterogeneity of NPQ during photosynthetic induction in these hypostomatous, heterobaric leaves that was adequately integrated by spot measurements, despite long-lasting artifacts from repeated saturating flashes during assays. Low NPQ in chloroplast containing cells adjacent to the mid vein, 1st and 2nd order veins defined areas of more dynamic NPQ large- and small-scale heterogeneity most strongly expressed in mesophyll cells between 3rd and 4th order veins. Both responded to external CO₂ concentration ([CO₂]) and to relative humidity, leaf dehydration, and occlusion of stomata with Vaseline™. Modulation of heterogeneous NPQ presumably reflected independent response of stomata in adjacent areoles that was largely expressed through CO₂ limited photosynthesis. Heterogeneity was most pronounced and prolonged in the absence of net CO₂ fixation in 100 ppm [CO₂] when respiratory and photorespiratory CO₂ cycling constrained the inferred rate of photosynthetic electron transport (ETR) to ~75 % of values in 400 or 700 ppm [CO₂]. Likewise, sustained higher NPQ under Vaseline™, after dehydration or at low RH, also restricted ETR to ~75% of controls. Low NPQ in chloroplast containing cells adjacent to major veins but remote from stomata suggests internal sources of high [CO₂] in these tissues.

Key words: avocado, heterobaric anatomy, patchy stomata, photosynthetic induction, Persea americana, xanthophyll pigments
INTRODUCTION

Long-lived shade leaves of avocado (García-Plazaola et al 2007) and many other tropical trees possess two xanthophyll cycles with distinctive kinetic properties that potentially both protect and promote the efficiency of light harvesting in the shade (Matsubara et al 2007, 2008, 2009). Functional evaluation of these properties required better understanding of the dynamics of non-photochemical quenching (NPQ) of chlorophyll fluorescence during photosynthetic induction at low light intensities. Spot measurements of photosynthetic electron transfer rates (ETR) using a pulse amplitude modulated (PAM) chlorophyll fluorescence instrument showed photosynthetic rates of avocado inner canopy shade leaves were 2 orders of magnitude slower than in the North-facing outer orchard canopies in Eastern Australia (Matsubara et al. 2012) and comparable to those of shade-grown avocado in the laboratory (Förster et al 2011, Jia et al 2013).

The sensitivity for detection of H₂O and CO₂ exchange in leaves of shade plants with such extremely low rates of photosynthesis is problematic and it was no surprise to find few data on the contribution of shade canopy photosynthesis to yield (Lui et al. 2002) or its sensitivity to the draconian pruning regimes sometimes applied in canopy management of this widely planted crop (Whiley et al. 2002). We needed to know if these parameters were uniform across the large thin leaves and whether the saturating flash used in these assays was intrusive, potentially altering parameters in the course of measurements. To this end, we compared estimates of ETR and NPQ with CO₂ and H₂O exchange measurements using a combined gas exchange-chlorophyll fluorescence system and a chlorophyll fluorescence imaging (CFI) system. Both systems were based on PAM technology, but had different detectors and excitation and actinic light sources.

In particular, we needed to check if properties measured by this technique were sensitive to stomatal closure. The contribution of patchy stomatal responses to oscillations
in photosynthesis had been successfully explored using CFI (Mott et al. 1993; Cardon et al. 1994; Seibke and Weis 1995a, b; Oxborough and Baker 1997). When combined with thermal imaging, the local effects of patchy stomatal closure on leaf temperature and metabolism have been demonstrated with remarkable precision (Omasa and Takayama 2003; West et al. 2005). Our preliminary CFI study revealed that transient, very heterogeneous NPQ in shade-grown avocado leaves during photosynthetic induction experiments was especially sensitive to occlusion of stomata with Vaseline™ (Takayama et al. 2008; c.f., Morrison et al 2005; Lawson 2009). We have expanded these imaging studies to illustrate how the duration, and the extent, of heterogeneous NPQ is sensitive to [CO₂], dehydration and RH, and is related to the heterobaric architecture of avocado shade leaves.

RESULTS

Slow photosynthesis in avocado shade leaves pushes the sensitivity limits of gas exchange; better data are obtained using chlorophyll fluorescence

Our initial objective was to discover how well gas exchange measurements were aligned with spot estimates of ETR from chlorophyll fluorescence and also whether the spot assays adequately integrated heterogeneity of NPQ previously observed in these leaves. In expt#1 three adjacent areas on a large detached leaf (Fig. 1 A) were first examined at three different [CO₂] at 25 ºC and 65% relative humidity in the LI-6400 40LCF gas exchange/chlorophyll fluorescence system. Following these measurements the same area of the leaf was examined by CFI using the Technologia system under the same. The saturating flash used in the LICOR system to monitor kinetics of ETR and NPQ at 30 s intervals during photosynthetic induction at 100 µmol photons m⁻² s⁻¹ was somewhat intrusive with these shade-grown leaves. The three spots examined in the LICOR system were still visible as areas with about 10% lower Fᵥ / Fₘ for up to 4.5 h after gas exchange measurements (Fig. 1B). The cover
picture shows another experiment in which lower NPQ in the spot interrogated by the saturating LICOR flash persisted for 20 min during subsequent imaging of the NPQ transient, but had disappeared in a 2nd CFI experiment the next day.

Gas exchange measurements were “noisy”, and tested the sensitivity limits of the system, even at the slowest practicable flow rates (Fig. 2). Net CO₂ assimilation remained negative throughout induction in 100 ppm [CO₂], reached ~1.0 µmol m⁻² s⁻¹ after 20 min in 400 ppm [CO₂], and saturated at ~ 2.0 µmol m⁻² s⁻¹ after 5 min in 700 ppm [CO₂] (Fig. 2 A). Stomata opened slowly (Fig. 2 B) but the extremely low conductance was responsive to [CO₂] (~25 mmol m⁻² s⁻¹, ~7 mmol m⁻² s⁻¹ and ~3 mmol m⁻² s⁻¹ in 100, 400 and 700 ppm [CO₂] respectively after 20 min). Consequently, estimates of intercellular [CO₂] in these leaves were dubious ([C₅₅] 100 ppm and intercellular [C₅₅] 126 ± 5 ppm; [C₅₅] 400 ppm: [C₅₅] 180 ± 13 ppm; [C₅₅] 700 ppm: [C₅₅] 101 ± 24 ppm) and of little use.

In contrast, chlorophyll fluorescence based estimates of ETR during induction at all three [CO₂] were much less “noisy” and were compatible with the slow rates of CO₂ assimilation (Fig. 2 C). Plots of ETR against net CO₂ exchange (Fig. 2 D) emphasised that after 20 min in 100 ppm [CO₂], ETR of 16 µmol m⁻² s⁻¹ supported little if any net CO₂ assimilation (Fig. 2 A) and presumably was consumed in photorespiratory CO₂ cycling. The small increase in ETR with time in 400 ppm supported slow but increasing net CO₂ fixation. In 700 ppm [CO₂], ETR was little different to that in 400 ppm [CO₂] but net CO₂ fixation increased to ~2 µmol m⁻² s⁻¹ despite further stomatal closure. After 5 min in 100 µmol photons m⁻² s⁻¹ the ratio of ETR in CO₂ limited (100 ppm) and saturated (700 ppm) treatments measured by chlorophyll fluorescence during gas exchange and by CFI was similar (~ 75%; Table 1).

In view of the particularly slow rates of photosynthetic CO₂ fixation and limited capacity for ETR in expt#1, it was not surprising to observe transiently higher NPQ (Fig. 3...
A) on transfer from dark to 100 µmol photons m⁻² s⁻¹. The transient was initially faster, larger and more prolonged at 100 ppm [CO₂] than in the other treatments and presumably arose from the lower ETR constrained by low external [CO₂] and concurrent photorespiratory CO₂ cycling. These NPQ transients during induction were confirmed by CFI measurements when repeated on the same leaf (Fig. 3 B). Good qualitative agreement was obtained between NPQ estimated by fluorescence data from the blue and red LED actinic illumination in the LICOR gas exchange system and values subsequently measured from the same spot after the same interval under the orange LEDs in the imaging system (Fig. 3 A). The correlation was highest in 400 ppm [CO₂] (R² = 0.967) but lower in the other treatments (R² = 0.847 in 100 ppm [CO₂]; R² = 0.304 in 700 ppm [CO₂]). Under these conditions and for these leaves, chlorophyll fluorescence and CFI clearly offered more convenient, more sensitive and reliable approaches to analysis of photosynthetic activities than gas exchange.

**Transient NPQ during photosynthetic induction is sensitive to external [CO₂]**

The imaging experiments revealed that the NPQ transients were accompanied by extensive heterogeneity. Illumination was most uniform in the central third of the large leaf, yet extensive but transient large-scale heterogeneity in NPQ was found at some time in all treatments (Fig. 3 B). Average NPQ imaged by CFI over the LICOR spots, and over the edited central area of the images shown in Fig. 3 B, were both highly and linearly correlated with the whole leaf image average of NPQ across all [CO₂] (LICOR spots R² = 0.978; edited central area R² = 0.999; Supplementary data 2). Evidently, average NPQ measured by CFI over the whole leaf and over the edited central area was not compromised by persistently lower Fv / Fm in the spots interrogated by the 40 saturating flashes from the LICOR system (Fig. 1 B). In addition, LICOR and CFI estimates of NPQ in expt#1 were similar to other measurements using the MINI-PAM system (Supplementary data 3).
Spatial variation in the extent and duration of NPQ during induction was observed at the three [CO₂] used in expt#1 (Fig. 3 B). Heterogeneity was already evident after 3 min at 400 ppm [CO₂], and the pattern as a whole persisted as NPQ declined after 7 min, but largely disappeared after 15 min. Some heterogeneity, with topography similar to that in 400 ppm [CO₂] after 7 min, appeared within 3 min in 700 ppm [CO₂], but had largely disappeared after 7 min. In contrast, heterogeneity only emerged in 100 ppm [CO₂] during relaxation from 7 min of sustained high NPQ (Fig. 3 B), and remained after 20 min. Overall, there was considerable consistency in the pattern and extent of heterogeneity between images obtained in 100 ppm [CO₂] after 15 min, in 400 ppm after 7 min, and in 700 ppm [CO₂] after 3 min. Comparisons of NPQ across the same X-Y transect with time showed large-scale heterogeneity of lower NPQ between the mid-rib and 1st order veins, as well as substantial small-scale heterogeneity within these areas (Fig. 3 C). Complex topography and variable kinetics of the transient heterogeneity in NPQ was a consistent feature of all leaves subsequently examined.

Large-and small-scale heterogeneity of transient NPQ is defined by leaf anatomy

Considerable within-leaf fidelity in the patterns of both large-scale and small-scale heterogeneity was observed during repeated imaging of photosynthetic induction on the same leaf, but there was substantial variation between leaves and experiments. The sequence of treatments at different [CO₂] was routinely varied to minimise, and to unravel, the possible after-effects of repeated measurements on the same leaf and to test relationships between heterogeneity and [CO₂]. For example, from gas exchange measurements in Fig. 2 B it seemed likely that commencing a series of observations on the same leaf with a pre-treatment in 700 ppm [CO₂] might minimize stomatal apertures and lock-in an atomically
determined pattern that would extend the duration of high, heterogeneous NPQ measured in subsequent exposures at lower $[\text{CO}_2]$. 

This hypothesis was confirmed in expt#2 where a highly stable pattern of heterogeneous NPQ persisted through three successive induction experiments on the same leaf (Fig. 4). Heterogeneity of NPQ peaked after 2 min in the first imaging series at 700 ppm $[\text{CO}_2]$, and almost precisely the same pattern of vein-defined large- and small-scale heterogeneity was evident after 1 min in a 2$^{\text{nd}}$ imaging series at 400 ppm $[\text{CO}_2]$ and after 5 min in a 3$^{\text{rd}}$ experiment at 100 ppm $[\text{CO}_2]$. However, when imaging was repeated in 400 ppm $[\text{CO}_2]$ following the 100 ppm assay the previous pattern of heterogeneity was abolished. It seemed likely that 20 min in 100 ppm $[\text{CO}_2]$ had re-opened stomata (c.f., Fig. 2 B), suggesting transient heterogeneity in NPQ might be controlled by differential responses of stomata throughout the leaf. As observed in expt#1, whole image ETR in 100 ppm $[\text{CO}_2]$ was also $\sim 75\%$ of that in 700 ppm treatments (Table 1).

Thick transverse sections of avocado shade leaves confirmed the potentially heterobaric leaf anatomy of avocado shade leaves. Although chloroplast containing palisade mesophyll cells extended over most of the upper surface of the mid-rib, thick walled cells without chloroplasts extended above and below the vascular tissues clearly making lateral gas exchange across the mid-rib unlikely (Fig. 5 $\text{top}$). Similar, but less massively thickened chloroplast-free vein extensions above and below 1$^{\text{st}}$ and 2$^{\text{nd}}$ order veins suggest that the lamina was heterobaric at these scales, and this might account for the large scale heterogeneity of NPQ. Such extensions were not obviously associated with 3$^{\text{rd}}$ and 4$^{\text{th}}$ order veins, where palisade mesophyll cells appeared to offer a path for lateral gas diffusion between adjacent areoles (Fig. 5 $\text{bottom}$). Small-scale heterogeneity of NPQ at this scale might be associated with variable, independent stomatal responses in adjacent areoles. Also, it is important to note that chloroplasts in cells adjacent to the upper vein extensions of the
mid-rib and 1st order veins were most distant from the stomata restricted to the abaxial epidermis of avocado leaves. Access to alternative internal sources of CO₂ could explain the consistently lower NPQ in the vicinity of these veins.

The extent and duration of transiently heterogeneous NPQ increases when stomata are occluded with Vaseline™ and following dehydration of leaves in air

If the transient heterogeneity in NPQ arose from differences in rates of stomatal opening and access to CO₂ in adjacent areoles we predicted that occlusion of stomata in larger patches of the leaf with a patch of Vaseline™ would generate higher and sustained NPQ in the treated areas compared to untreated areas. There are no stomata on the adaxial epidermis of avocado leaves and treatment of the upper epidermis with Vaseline™ had no effect on the transient in NPQ or its heterogeneity. Occlusion of stomata with a patch of Vaseline™ applied to the abaxial epidermis in expt#3 rapidly led to sustained higher NPQ in the treated area during induction experiments (Fig. 6 A), whereas vein-defined heterogeneity of NPQ in untreated areas peaked within 3 min and disappeared after 15 min (Fig. 6 B). Estimates of ETR in the Vaseline™ treated area were 79% of those in adjacent untreated areas (Table 2) suggesting occlusion of stomata constrained access to CO₂ and depressed ETR in air to about the same extent as previously observed under 100 ppm [CO₂] (Table 1).

Image transects at 3 min showed six clearly defined “high amplitude” peaks of NPQ small scale heterogeneity were present between the low values associated with the 1st order veins in the untreated section (Fig. 6 C). Whilst NPQ was higher in the treated area, fewer much more damped peaks were evident either side of these veins. Small-scale, high-amplitude heterogeneity in adjacent areoles had evidently coalesced under Vaseline™. Interestingly, areas close to the 1st and 2nd order veins within the treated area showed
markedly lower NPQ than the bulk mesophyll between veins, and this area expanded with time (Fig. 6 D).

Water supply to the Vaseline™ treated leaf was removed and the leaf was allowed to dehydrate slowly in the dark for 8 h before repeating the imaging experiment under the same conditions (Fig. 6 E, F). The sharp distinction in NPQ between the Vaseline™ treated and untreated control areas of the leaf disappeared following dehydration. Image transects of NPQ in control areas following dehydration revealed two-fold higher values after 3 min induction (Fig. 6 G) and these did not decline much over 15 min (c. f. Figs. 6 D and H). The overall pattern of heterogeneity in the transient was amplified by dehydration, and it was clear that the fine scale heterogeneity initially evident in control areas tended to coalesce to areas of higher NPQ after dehydration. In contrast, dehydration had a much smaller effect on the treated area of the leaf. Compared with initial values imaged after 3 and 15 min, NPQ in the inter-vein areas treated with Vaseline™ actually declined during dehydration and the decline in NPQ continued to spread out from the main veins with time.

**Heterogeneity of NPQ during dehydration responds to [CO₂]**

Patterns of heterogeneous NPQ during dehydration in air mimicked those arising from occlusion of stomata with the Vaseline™ so the question arose as to whether high [CO₂], which itself closed stomata compared to air (Fig. 2 B), might modify this response. In expt#4, slow dehydration in 400 ppm [CO₂] for 6 h led to an increase in the magnitude and duration of transient NPQ during photosynthetic induction (Fig. 7 A). Whole image estimates of ETR after 5 and 20 min averaged 75% of the control (Table 2) suggesting dehydration in air closed stomata as effectively as Vaseline™ treatment. Compared to the 400 ppm [CO₂] treatment, the control induction in another leaf at 700 ppm [CO₂] (expt#5) produced initially higher NPQ with marked heterogeneity after 2 min (Fig. 7 B), presumably
because high [CO₂] itself reduced stomatal conductance (Fig. 2 B). The level of NPQ declined but heterogeneity became more obvious after 5 min and had disappeared after 20 min as ETR increased (data not shown). Curiously dehydration produced initially lower overall NPQ with greater heterogeneity (Fig. 7 B) at similar ETR, but as in 400 ppm [CO₂], dehydration extended the duration of the transient and maintained heterogeneity. As before, ETR in the dehydrated leaf after 5 and 20 min was 77% of the control (Table 2), suggesting stomata were more tightly closed after dehydration.

Two changes in the patterns of heterogeneity in NPQ of dehydrated leaves were noted in Figs. 6 and 7. First, although decline of NPQ during the transient was always faster in cells closer to major veins than in bulk mesophyll, this contrast was strongly enhanced by dehydration under both [CO₂]. Second, fine-scale heterogeneity in NPQ that was initially defined by 3rd order veins in controls was lost after dehydration, especially in 400 ppm [CO₂]. Comparisons of the same area of interest before and after dehydration showed areas of differing NPQ initially separated by 3rd order veins had coalesced (Fig. 7 C). Stomatal closure in adjacent areoles during dehydration may have allowed equilibration of [CO₂] between these compartments. Palisade mesophyll cells above the 3rd order veins seemed less clearly isolated by chloroplast-free vein extensions (Fig. 5).

**Transient heterogeneity of NPQ responds to interactions between [CO₂] and RH**

Stomatal responses to external [CO₂] may interact with atmospheric humidity, and if development of transient heterogeneity in NPQ was under stomatal control in adjacent areoles, these features might also respond to differences in relative humidity. To this end, expt#1 was repeated several times at 25% and 60% relative humidity. The time course of transients in average whole image NPQ to [CO₂] (Fig. 8 A and B) were similar to those previously measured by gas exchange and imaging (Fig. 3 A), but there was large variation
between experiments on different leaves. Low RH seemed to prolong the slow, transiently higher NPQ in 100 ppm [CO₂] and to accelerate the fast, transiently lower NPQ in 700 ppm [CO₂]. High variability in 400 ppm [CO₂] precluded any clear response.

There was some indication of differing effects of RH and [CO₂] on large- and small-scale heterogeneity. One set of CFI data (expt#6) from the same leaf is shown in Fig. 8 C. Low RH seemed to preclude the emergence of large-scale heterogeneity of NPQ that emerged in the circled central areas of the leaf under 60% RH, regardless of ambient [CO₂]. This was reminiscent of the CO₂ and time dependent NPQ observed in expt#1 (Fig. 3 B). Transect data (black lines) in 400 ppm [CO₂] images (Fig. 8 E) contrast the sustained higher NPQ with small-scale heterogeneity at 25% RH with the persistent large-scale heterogeneity at 60% RH. Compared to 60% RH, low humidity drastically reduced initial ETR in 100 ppm and 400 ppm [CO₂] (Table 2), consistent with more closed stomata in 25% RH. As observed in expt#1 (Fig. 2 B), in expt#6 whole image ETR in 700 ppm [CO₂] was identical in both 25 and 60 % RH, and was the same as 60% RH with 400 ppm [CO₂] (data not shown). More experiments, combining more sensitive gas exchange systems, spot measurements of chlorophyll fluorescence and CFI, are needed to test these complex interactions in avocado shade leaves.

**DISCUSSION**

The shade-grown leaves of avocado studied here represent a special case of exceedingly slow photosynthesis in long lived leaves that tested the limits of measurement in a portable gas exchange system. Under these conditions and for these large leaves, chlorophyll fluorescence spot measurements potentially offered a more convenient, more sensitive and reliable approach to analysis of photosynthetic activities. However, we needed to discover whether heterogeneity in photosynthetic parameters, if it occurred, would compromise spot
measurements. We also needed to know if different excitation and detection methods used by the LICOR 40LCF and Technologia CFI systems for chlorophyll fluorescence measurements (with some differences in parameter calculation) delivered similar results. Our comparison of spot measurements and CFI methods exposed potential artefacts arising from repeated saturating flashes during spot measurements but revealed spectacular images of anatomically defined, transiently heterogeneous NPQ during photosynthetic induction.

In retrospect, we erred in seeking to achieve comparable yields of chlorophyll fluorescence from the two systems, and to obtain high time resolution in gas exchange and fluorescence measurements. This led to a “saturating flash photon load” that delivered twice the actinic exposure to the spot interrogated for fluorescence in the LICOR gas exchange curvet, and added 50% to the actinic exposure to the whole leaf during Technologia CFI imaging. We should not have been surprised by the “after-glow” spots of lower ∆F/Fm’ and initially higher NPQ that were revealed by the imaging system to persist for some hours after induction analyses using frequent saturating flashes in the LI-6400 40LCF (Fig. 1 B and cover picture).

Although these observations highlighted the care needed in selection of instrument settings for chlorophyll fluorescence spot measurements with shade leaves, they did not seriously compromise the comparison of the two approaches. Whole leaf image average NPQ was highly correlated with image average NPQ over the spot interrogated by LI-6400 40LCF and with the edited area of the image at all [CO₂] tested (Supplementary data 2), despite the large range of NPQ values in the CFI data (Fig. 3). Qualitatively good agreement between NPQ kinetic data from spot measurements and images of NPQ in edited central area was reassuring, especially in 400 ppm [CO₂]. The disparities between the two data sets in 100 ppm and 700 ppm [CO₂] (Fig. 3 A) may have been influenced by the measurement sequence.
The spectacular images of transiently heterogeneous NPQ in avocado shade leaves during photosynthetic induction at 100 µmol photons m$^{-2}$ s$^{-1}$, and its relationship to leaf anatomy became the main focus of the study and are discussed in three related contexts:

(i) static factors associated with leaf vascular morphology that created small- and large-scale patterns in heterogeneity of NPQ,

(ii) dynamic factors that modulated this heterogeneity with time and PFD and were evidently dominated by heterogeneous responses of stomata in adjacent areoles defined by the leaf morphology and

(iii) memory effects that emerged within both of the above contexts and responded to the limited photosynthetic metabolism under the conditions examined. In addition, we examine potential connections between the transiently heterogeneous NPQ and the two xanthophyll cycles in these leaves (Förster et al 2009).

In brief, we believe the first two of the above are manifested in heterobaric leaf anatomy, and reflect transient, localized exposure of the photosynthetic apparatus to excess light, even in 100 µmol photons m$^{-2}$ s$^{-1}$. This led to heterogeneous development of NPQ early in photosynthetic induction that was responsive to external [CO$_2$], relative humidity, dehydration and was remarkably sensitive to occlusion of stomata with Vaseline™. All of these variables are well known to influence stomatal aperture (Lawson et al. 2002; Lawson 2009; Mott 2009). The memory effects (Fig. 4) that persisted for an hour in the dark between repeated imaging of induction under different [CO$_2$] were especially interesting, and may be a consequence of the generally low stomatal conductance. Images of transient heterogeneity of NPQ evidently serve as especially responsive surrogates, reporting metabolic responses to stomatal control of access to external [CO$_2$], dehydration and low humidity in adjacent areoles, within the overall constraints of vascular architecture (Downton et al. 1988; Terashima et al. 1988; Cardon et al 1994; Meyer and Genty 1999).
Large-scale heterogeneity was usually delineated by lower NPQ over 1st and 2nd order veins with strong, chloroplast-free vein extensions to the upper and lower epidermis. Such heterogeneity appeared at different times in different external [CO₂] treatments (Figs. 3 B and 8 B) and persisted between treatments (e.g., Fig. 4) and after occlusion of stomata with Vaseline™ (Fig. 6). It remained clearly delineated by lower NPQ in the vicinity of major veins. Low NPQ in palisade mesophyll cells above major veins was surprising because these cells are furthest from stomata on the lower epidermis and it was expected that these cells would show high NPQ due to limited access to CO₂. Moreover, NPQ adjacent to 1st and 2nd veins declined more rapidly with time under Vaseline™, and during dehydration lower NPQ appeared to spread from major veins with time. On the other hand, it is possible that chloroplast containing cells adjacent to major veins enjoy better supply of CO₂ from internal sources. These might include diffusion into the xylem water stream from adjacent areas of the leaf and release during translocation through areas with occluded stomata, or respiratory CO₂ generated from metabolically active phloem cells during sugar uptake and translocation (Siebke and Weis 1995b). Image analyses revealed low NPQ adjacent to major veins was accompanied by measurably higher ETR, consistent with better CO₂ supply (data not shown). Striking, images of reduced [O₂] and heightened respiration in the vicinity of veins in leaves (Tschiersch et al. 2012) suggest the above explanation is plausible. It may be relevant that in developing sink leaves of tropical trees photosynthetic activity first appeared adjacent to major veins (Walter et al. 2004).

Small-scale heterogeneity associated with 3rd and 4th veins was much more dynamic. Third order veins usually had some thick-walled, chloroplast-free cells above and below the vascular tissues whereas such tissues were rudimentary or absent above 4th order veins. These veins had the potential to define areola with limited horizontal gas exchange connectivity, leading to variations in the kinetics of NPQ, driven by access to CO₂.
determined by independent responses of stomata in adjacent areoles. Dehydration caused small-scale heterogeneity to coalesce (Figs. 6 and 7).

Previous bench-mark studies of spatial heterogeneity in chlorophyll fluorescence parameters used leaves with high photosynthetic capacities and dealt with stomatal and/or metabolic oscillations precipitated by sudden changes in PFD, [CO₂] or humidity during steady state photosynthesis (Mott et al. 1993; Cardon et al. 1994; Siebke and Weis 1995a, b; Bro et al. 1996). Although the duration and amplitude of the single transient in NPQ observed here on transfer from dark to 100 µmol photons m⁻² s⁻¹ responded to [CO₂], it showed no tendency to oscillation.

The exceptionally low values of stomatal conductance (~ 25 mmol m⁻² s⁻¹) in avocado shade leaves studied here are similar those reported for attached avocado sun leaves in the laboratory (76 mmol m⁻² s⁻¹; Mickelbart et al. 2000) and under high vapor pressure deficits in the field (100 mmol m⁻² s⁻¹; Whiley et al 2002). The absence of net CO₂ assimilation and maximum stomatal conductance in 100 ppm [CO₂] implied that gas exchange was dominated by respiratory and photorespiratory CO₂ recycling and net CO₂ efflux. An early estimate (Osmond 1981) placed the energy demand of C₃ photosynthesis at CO₂ compensation at 60-70 % of that in air. The ETR in 100 ppm [CO₂] averaged only 75% of that in air (400 ppm [CO₂]), and 700 ppm [CO₂], whether measured by spot or imaging assays (Table 1). Interestingly, when stomata were occluded by Vaseline™ in air, and when stomata were presumed to close during dehydration or in response to low RH, ETR measured as average whole leaf CFI was also ~75% of controls (Table 2). Evidently, metabolic constraints on utilization of ATP and NADPH under these conditions contributed to variations in transient heterogeneity of NPQ in all of these situations.

Photosynthetic control of ETR (Foyer et al. 1990) by carbon limited metabolism and constrained utilization of ATP and NADPH would be expected to produce a higher ΔpH in
chloroplasts during induction and promote higher, more prolonged transient NPQ in 100 ppm [CO$_2$] (Horton et al. 1986; Meyer and Genty 1999) in which excess excitation is dissipated as heat in light harvesting antenna. Evidently these detached shade-grown leaves commonly experienced excess excitation in the early stages of photosynthetic induction in 100 µmol photons m$^{-2}$ s$^{-1}$. The sun fleck environments of avocado inner canopies are not well known. Nichol et al. (2012) cited representative measurements at midday in Californian orchards of from 7 min to 20 s at ~120 to ~150 µmol photons m$^{-2}$ s$^{-1}$ on backgrounds of 4 to 18 µmol photons m$^{-2}$ s$^{-1}$, respectively (M. V. Mickelbart; personal communication). These observations suggest the transiently heterogeneous NPQ produced in the experiments described here, may occur under natural conditions, but further close study is needed to place these processes in context.

From information available at the time, the first report of transient heterogeneity of NPQ in these leaves Takayama et al. (2008) proposed that xanthophyll-independent, ∆pH-dominated processes were involved. We now know that de-epoxidation of V to A and Z (DESV increased from 0.04 ± 0.02 to 0.25 ± 0.03; n = 6) occurred during the imaging assays used here, but we do not know if it was coincident with the transient during photosynthetic induction at 100 µmol photons m$^{-2}$ s$^{-1}$. Xanthophyll based stabilization of NPQ is generally more strongly expressed in sun than in shade leaves (Demmig-Adams 1999). Relationships between fast relaxing NPQ$_{∆pH}$ and slow relaxing NPQ$_{AZ}$ (Jia et al 2013) remain poorly understood. However, de-epoxidation of Lx to L was not detected in the above experiments, so if accumulation of Lx promotes efficiency of light harvesting (Matsubara et al 2007; 2011), this feature would not be compromised during the NPQ transients reported here. Further evaluation of these processes in avocado shade leaves is proceeding using the much less intrusive LED induced fluorescence transient (LIFT) system (Nichol et al 2012).
Acknowledgements

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Conflicts of interest: There are no conflicts of interest. Figure 6 A is part of a whole leaf image that appeared in black and white in Takayama et al (2008) and in color collage in the review of Nichol et al (2012). The cover image accompanying Jia et al. (2013) was prepared by Takayama and Osmond from other experiments not reported here.

MATERIALS AND METHODS

Plant material

Seedlings (~60 cm tall) of avocado (*Persea americana* L., cv Edranol) were purchased from Vallance’s Nursery (Mullumbimby NSW 2482 Australia) and maintained in 8 L containers of potting soil with regular irrigation and additions of slow release nutrients in a shaded section of a temperature controlled glasshouse for 12 months (18°C night/ 29°C day).
Plants were pruned to the main stem 6 months prior to experiments and fully expanded attached and detached leaves (20-35 cm length) from the second flush of new growth were examined close to the southern Spring equinox (August-September 2006). The light environment at midday (measured with a LI-190 sensor; www.licor.com) was ~ 50 µmol photons m\(^{-2}\) s\(^{-1}\) in the upper canopy and ~ 20 µmol photons m\(^{-2}\) s\(^{-1}\) in the lower canopy of the neutral beige shade cloth enclosure when full sun intensity in the unshaded greenhouse was 1600 µmol photons m\(^{-2}\) s\(^{-1}\). Leaves from plants in the shade enclosure were detached by cutting the petiole under water and sealing the leaf into a microfuge tube water reservoir with Parafilm™. Leaves were wrapped in moist paper towels and quickly transferred to the laboratory in a styrofoam container to avoid exposure to light and to minimize exposure to low temperature. These leaves gave reproducible results in gas exchange and imaging systems for at least 24 h.

**Gas exchange measurements**

Photosynthetic gas exchange of detached leaves was measured in the small curvet of a LI-6400 40LCF system (LICOR Inc, Lincoln NE, USA) fitted with a PAM chlorophyll fluorescence detector. Measurements in expt#1 were made on three adjacent but well separated areas in the central third of a single large, fully induced shade leaf harvested mid-afternoon. After 30 min dark adaptation photosynthetic induction was followed for 20 min in 100 µmol photons m\(^{-2}\) s\(^{-1}\) (with saturating flashes at 30 s intervals) in atmospheres of 100, 400 (air) and 700 ppm [CO\(_2\)] at 65% at flow rates of 0.2 ml min\(^{-1}\) to maximize sensitivity. Saturating flashes were delivered to a small disc on the leaf in the curvet for simultaneous measurement of chlorophyll fluorescence parameters. The spots exposed to saturating flashes were marked for subsequent reference in the imaging system. Calculation of ETR and NPQ in the LICOR followed the convention of Genty et al (1989) i.e., $ETR = \frac{\Delta F}{F_m'} \times PFD \times 0.85 \times 0.5$ and $NPQ = \frac{F_m}{F_m'} - 1$. 

*PFD*0.85*0.5 and $NPQ = \frac{F_m}{F_m'} - 1$. 

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Chlorophyll fluorescence imaging protocols

The same parameters were measured on the same area of the same leaf using the Technologica CF Imager system with Fluorimager Two software (www.technologica.co.uk; Oxborough and Baker 1997; Barbagallo et al. 2003). After refreshing the water supply the leaf was transferred to a small plexiglass treatment chamber within the imaging system (Supplementary data 1) with controlled air flow (~7 L min\(^{-1}\)) that delivered the same [CO\(_2\)] and RH treatments to the leaf as used in the LICOR. Fluorescence was imaged from the almost planar upper epidermis; images from the lower surface revealed qualitatively similar patterns, but the raised vascular elements tended to introduce optical artefacts. The leaf was kept in 400 ppm [CO\(_2\)], 65% RH, 25 °C for 2.5 h dark adaptation before imaging photosynthetic induction curves on the whole leaf in saturating flashes at zero time, after 20 and 50 s, then after 2, 3, 5, 7, 10, 15 and 20 min, followed by measurements at intervals up to 6 min in the dark. The 400 ppm [CO\(_2\)] images were obtained 4 h after gas exchange measurements, 700 ppm [CO\(_2\)] images after 3 h whereas the images in 100 ppm [CO\(_2\)] were taken 7 h after gas exchange. In this and all subsequent imaging experiments leaves were loosely fixed in the CFI system gas exchange chamber with adhesive tape to allow free gas exchange to stomata on the lower epidermis.

The differing saturating flash and actinic light sources and detectors in these systems made it very difficult to produce equivalent fluorescence yields from the areas of interest, and efforts to do so were misplaced. The saturating flash settings used in the LICOR (0.8 s at 7660 µmol photons m\(^{-2}\) s\(^{-1}\)) were substantially higher those achievable with optimal uniformity over the large leaves in the Technologia system (0.8 s at 5,370 µmol photons m\(^{-2}\) s\(^{-1}\)) yet the small area of the spot measurement produced somewhat lower values for F\(_{m}\) (2,547 ± 22) than the large area surveyed by the Technologia CFI (3,326 ± 41). The accumulated photon load during the 30 s spacing of the saturating flashes of the LICOR protocol delivered about twice the actinic load whereas the accumulated photon load of the
widely spaced saturating flash protocol of the CFI protocol was only half that of the actinic load. Moreover, although the calculation of chlorophyll fluorescence parameters in the LICOR and Technologia systems followed the same principles, values for NPQ presented by the imager software and used to construct image transects were some 70% greater than those calculated from the raw chlorophyll fluorescence data accompanying the images. In addition, estimates of whole image ETR from CFI in this experiment were 20 to 40% lower than those estimated in the LICOR system. Values for NPQ (and ETR) in other avocado shade leaf experiments using optimised assays in the MINI-PAM system (H.Walz, Effeltrich DE; Förster et al 2011) matched those in the LICOR system (Supplementary data 3). Changes in pigment composition in discs punched from leaves before and after the standard induction curve were assayed as described by Förster et al (2009).

**Analyses of heterogeneity in NPQ images**

In expt#2 the controlled atmosphere chamber was used to facilitate CFI analysis of a persistent pattern of large scale heterogeneity throughout the sequence of 700, 400 and 100 ppm [CO₂], but which was abolished following a return to 400 ppm [CO₂]. The likelihood that heterogeneity of NPQ in expt#1 and expt#2 was due to patchy response of stomata to internal and external [CO₂] was examined in expt#3 by carefully covering a small area of the lower epidermis with Vaseline™ to restrict CO₂ exchange. The water reservoir was removed after the initial CFI to allow observation of NPQ and ETR following partial dehydration. Expt#4 and expt#5 repeated the dehydration responses in expt#3 by experiments in 400 ppm and 700 ppm [CO₂], respectively. The controlled environment was used in several other experiments to explore the transient heterogeneity of NPQ and its interactions with [CO₂] and RH. As in expt#1 the NPQ values in different experiments were calculated from raw chlorophyll fluorescence data in the imaging system during the transients, but NPQ values shown in the images the transects were those presented by imager software.
Quantitative analysis of NPQ data in expt#3 and subsequent experiments was achieved following export from the FluorImager program into ArcGIS 9.2 (ESRI 2009) via a file conversion in ENVI 4.8 (Exelis Visual Information Solutions 2010). Spot measurements were made by constructing suitably sized sampling polygons over areas of interest to confirm that heterogeneity was not scale dependent (data not shown). Transects of NPQ were established by creating lines across sequential images from each experiment in which the leaf was not moved between treatments. This produced transects for all equivalent images allowing for direct comparison across time periods and between other treatments. As leaves contracted during dehydration experiments, transects were adjusted to allow for changes in size between treatments. Points were constructed along each line, at a distance of 1 pixel, and the raw NPQ value for each point was obtained for each image. These data were then exported to Excel to calculate and present a transect of mean NPQ based on three adjacent pixels.

Leaf anatomy

Thick freehand transverse sections of avocado leaves were examined in a light microscope at x100 magnification. Images were collected in dark field mode using a 10x 0.3NA objective and a Spot Flex Camera (Diagnostic Instruments, MI USA) on a microscope system comprising an Olympus BX41Microscope (Olympus Optical, Japan), a CytoViva high resolution illuminator (CytoViva Inc, AL USA) with an EXFO X-Cite120 XL light source (EXFO Life Sciences, Canada). Avocado leaves were hypostomatous with numerous small stomata restricted to the lower epidermis (400-700 stomata mm\(^{-2}\); Scholefield and Kriedemann 1979; Mickelbart et al. 2000), and appeared to be heterobaric to at least 3rd order veins. Several replica techniques were used to detect whether the small stomata were open or closed, but without success, presumably because of the extensive wax deposits on adjacent epidermal cells (Mickelbart et al. 2000).
References


patchiness for a single surface of *Xanthium strumarium* L. leaves observed with

Table 1: Photosynthetic electron transport rates (ETR) in detached shade grown avocado leaves in 100 µmol photons m\(^{-2}\) s\(^{-1}\) measured using different chlorophyll fluorescence assays with limiting (100 ppm) or saturating (700 ppm) [CO\(_2\)].

<table>
<thead>
<tr>
<th>Assay method</th>
<th>ETR, µmol m(^{-2}) s(^{-1})</th>
<th>A/B %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A) [CO(_2)] 100 ppm</td>
<td>(B) [CO(_2)] 700 ppm</td>
</tr>
<tr>
<td>LI-6400 40LCF; spot (expt#1)</td>
<td>9.9</td>
<td>13.0</td>
</tr>
<tr>
<td>Technologia CFI; whole image (expt#1)</td>
<td>6.1</td>
<td>9.0</td>
</tr>
<tr>
<td>Technologia CFI; whole image (expt#2)</td>
<td>5.6</td>
<td>7.2</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Average whole image photosynthetic electron transport rates (ETR) in detached, induced shade grown avocado leaves after 2 to 7 min in 100 µmol photons m^{-2} s^{-1} measured by CFI during treatments designed to produce open or closed stomata.

<table>
<thead>
<tr>
<th>Experiment, [CO₂], time of images and treatments</th>
<th>ETR, µmol m^{-2} s^{-1}</th>
<th>A/B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A) Putative closed stomata</td>
<td>(B) Putative open stomata</td>
</tr>
<tr>
<td>Expt#3 [CO₂] 400 ppm; 5 and 7 min; A, treated with Vaseline™; B, control</td>
<td>9.1</td>
<td>11.6</td>
</tr>
<tr>
<td>Expt#4 [CO₂] 400 ppm; 5 min; A, after dehydration; B, control</td>
<td>6.6</td>
<td>8.9</td>
</tr>
<tr>
<td>Expt#5 [CO₂] 700 ppm; 5 min; A, after dehydration; B, control</td>
<td>5.7</td>
<td>7.4</td>
</tr>
<tr>
<td>Expt#6 [CO₂] 100 ppm; 7 min; A, 25% RH; B, 60% RH</td>
<td>6.7</td>
<td>11.1</td>
</tr>
<tr>
<td>Expt#6 [CO₂] 400 ppm; 7 min; A, 25% RH; B, 60% RH</td>
<td>8.6</td>
<td>11.7</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>72.8 ± 3.3</td>
<td></td>
</tr>
</tbody>
</table>
Figure legends

Figure 1: (A) Locations of three adjacent areas on the avocado leaf interrogated by the LICOR LI-6400 40LCF combined gas exchange/chlorophyll fluorescence system and the Technologia CF1 chlorophyll fluorescence imaging system in expt#1. The circled discs show the areas subjected to repeated saturating flashes in the LICOR. (B) The effects of the saturating flashes were still evident as areas of lower Fv/Fm in the image from the Technologia system 4.5, 4.0 and 3.3 h after gas exchange measurements in 100, 400, and 700 ppm CO₂, respectively.

Figure 2: Photosynthetic induction curves on the shade-grown avocado leaf in expt#1 as shown in Fig. 1 measured at 100 µmol photons m⁻² s⁻¹ at three different [CO₂] using the LICOR LI-6400 40LCF. The measurement sequence of 100, 400, and 700 ppm [CO₂] was chosen in the expectation that stomata would open most rapidly in the lowest [CO₂] and would thus be less likely to compromise subsequent interpretations of data at higher [CO₂]. (A) net CO₂ assimilation, (B) stomatal conductance and (C) photosynthetic electron transport rate (ETR). Data from (A) and (C) were replotted in (D) to emphasise that ETR did not support net CO₂ assimilation in 100 ppm CO₂ but rather was consumed in photorespiratory CO₂ recycling.

Figure 3: Analysis of transient large-scale heterogeneity of NPQ during photosynthetic induction in expt#1. The same three adjacent spots selected for assay at different [CO₂] (Fig. 1 A) were examined using both LICOR LI-6400 40LCF and Technologia CFI systems. (A) Comparative measurements of NPQ kinetics in the gas exchange system (open symbols; measurement sequence 100, 400, 700 ppm) and subsequently in the imaging system (closed symbols; measurement sequence 400, 700, 100 ppm) that followed 4 h, 5.3 h, and 14 h after
gas exchange measurements. (B) Selected images of NPQ in the central part of the leaf that illustrate the heterogeneity emerging with different kinetics at different [CO₂]. (C) Transects of NPQ (X to Y) across the mid-rib and two major veins (arrows). Note that NPQ values in (A) were calculated from image raw fluorescence data whereas data in (B) and (C) were those presented by imager software (see Materials and Methods).

Figure 4. Fidelity in the pattern of heterogeneity and the memory effect during transient NPQ in avocado shade leaves in expt#2. Successive induction curves from the same leaf were examined sequentially at 700, 400 and 100 ppm [CO₂], separated by 1 h in the dark under the preceding [CO₂]. The images were selected at times with closely comparable whole image NPQ. Stomatal opening in the third treatment with 100 ppm CO₂ evidently abolished the pattern of large-scale heterogeneity observed in the first three transients.

Figure 5. Thick transverse sections of an avocado shade leaf observed in dark field mode using a high resolution illuminator at x 100 magnification to show chlorophyll containing cells extending either side of a column of thickened cells over the mid-vein (top) and then in the vicinity of and between 1st, 2nd, 3rd and 4th order veins (bottom). There is little evidence for columns of thickened above 3rd and 4th order veins. All sections were taken from the same leaf and precision calliper measurements between 3rd order veins at leaf margins gave an average leaf thickness of 148 ± 8 µm.

Figure 6. Occlusion of stomata on the underside of a detached P. americana leaf with a patch of Vaseline™ (Vas) in expt#3 leads to rapid (A) and sustained (B) increase in NPQ at 400 ppm CO₂. Image transects of NPQ (white lines) show large scale heterogeneity between 1st order veins through the treated and untreated areas (C) that persists in the former but relaxes in the latter (D) with lowest NPQ over the veins (red triangles; black arrows). Small
scale heterogeneity appears as “noise” between these veins. Vaseline™ treatment mimics a quantitatively similar high NPQ that occurs over the whole leaf after 8 h dehydration in laboratory light (E) as leaf fresh weight declines by 29%. After dehydration NPQ relaxes more slowly in control areas, but faster in Vaseline™ treated areas (c.f., A and B vs E and F) and transect data (G and H) show the extent of coalescence of heterogeneity in treated and untreated areas.

Figure 7. Dehydration in expt#4 altered the extent, duration and form of heterogeneity during transients. (A) In 400 ppm CO₂ adjacent areola initially defined by 3rd order veins coalesced to larger areas after dehydration (fresh weight declined by 24%), presumably following stomatal closure. (B) Similar results were obtained in expt#5 during dehydration of another leaf in 700 ppm (fresh weight declined by 23%). (C) Enlarged areas of interest from images at 2 min before and after dehydration in 400 ppm CO₂ in (A) show coalescence of small scale heterogeneity in more detail.

Figure 8. Transiently heterogeneous NPQ responds to interactions between [CO₂] and RH. (A-C) Whole image NPQ calculated from raw fluorescence data (means ± SE; n = 2 to 4 experiments). (D) Selected NPQ images with time in the same leaf (expt#6) under three [CO₂] at high and low RH. Circles in 60% RH treatments identify a persistent patch of large scale heterogeneity that responds more rapidly with increasing [CO₂]. (E) Transects (black lines) after 2 and 10 min in 400 ppm [CO₂] show less change in heterogeneity with time at 25% RH whereas large scale heterogeneity increases markedly with time in at 60% RH.
Fig 3

A

B

C

Fig 4
Fig. 8
Supplementary data 1: Controlled atmosphere environment chamber for detached avocado shade leaves in the Technologica imaging system.

Supplementary data 2: Average values for NPQ in images at three different scales of analysis in Fig. 2. Average NPQ in the most evenly illuminated central portion of the leaf (Edited) and from the 3 different spots measured in the LICOR system (Spot) were very highly correlated with values for the whole leaf image.
Supplementary data 3: Comparison of NPQ during induction in avocado shade leaves at 100 \( \mu \text{mol photons m}^{-2} \text{ s}^{-1} \) by Technologia CFI imaging (NPQ image) and LICOR LI-6400 40LCF in this paper (16 September 2006) and using MINI-PAM (05-07 July 2009; \( n = 9 \)).